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POLARIMETRIC DETERMINATION OF STARCH IN CEREAL PRODUCTS

IV. CRITICAL STUDIES OF METHODS FOR THE DETERMINATION OF STARCH IN WHOLE WHEAT, GRANULAR, AND PATENT FLOURS[1]

BY K. A. CLENDENNING[2]

Abstract

A rapid polarimetric method applicable to whole wheat, granular, and patent flour is described in which the starch is dissolved in hot calcium chloride solution under uniform conditions, soluble proteins being removed by the addition of stannic chloride or uranyl acetate prior to volume adjustment, filtration, and polarization.

Approximately 2.5-gm. samples (40 mesh) are placed in tall beakers and suspended uniformly in 10 cc. of water. Calcium chloride solution (60 cc., d. 1.30, pH 5.5) and 2 cc. of 0.8% acetic acid are added. The mixture is boiled at constant salt concentration for 15 min., foaming being prevented by dropwise addition of *n*-octanol as required. After cooling, 2.5 to 5.0 cc. of 4% stannic chloride or 10 cc. of 5% uranyl acetate (each dissolved in calcium chloride solution) is added, the mixture is diluted to 100 cc. with calcium chloride solution, mixed, and filtered. After discarding the first portion, the filtrate is polarized in 2 dm. tubes. $[\alpha]_D = +203°$ is used in the calculations; % starch = 2 dm. reading in degrees $\times$ 10 when 2.463 gm. flour samples are employed.

Stannic chloride is shown to depress the specific rotation value of starches. A large error is introduced when it is used on wheat flour in the amount prescribed by Mannich and Lenz. Using the above uranyl acetate treatment, starch may be rapidly determined in corn, barley, rye, rice, grain sorghum, and buckwheat by the calcium chloride polarimetric method. It is shown that interfering wheat proteins may be removed by preparatory extraction with aqueous ethanol and dilute sodium hydroxide, as well as by precipitation. The latter being more rapid is judged most suitable for routine applications. Levorotatory hemicelluloses of bran and endosperm are dissolved in considerable amount by boiling calcium chloride solution but their small effect is balanced by the error of overestimation arising from insoluble wheat solids. Hemicelluloses are shown to be capable of causing high results in applications of the diastase–hydrochloric-acid method to wheat products. The improved calcium chloride polarimetric method is considered the most reliable of existing macromethods for the determination of starch in wheat.

Introduction

Methods for the direct determination of starch in complex products such as the cereal grains have been reported upon and used for more than 100 years. The earliest method appears to have been that of Hermbstadt (15), which was really a starch manufacturing operation conducted on a miniature

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as paper No. 26 on the Industrial Utilization of Wastes and Surpluses and as N.R.C. No. 1325.

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scale. Fuss made the first recorded attempt to determine starch in North American wheat, data upon which appeared in 1832 (14). Expressed in terms of total solids, his values range between 61.8 and 63.2%, the corresponding figures on 13.5% moisture basis being 53.5 and 56.4%. Hydrolytic and iodometric methods were described before 1850 and the optical rotatory power of starch solutions was reported upon by Biot and his colleagues (5, 31) in this same early period. Under Liebig's direction, Krocker (23) developed a rather ingenious fermentation method for the determination of "true" starch content. The data that this worker published in 1846 on all of the common cereal grains and legume seeds are, with but few exceptions, in good agreement with existing knowledge. Beyond the confusion that has arisen from calculations based on "nitrogen-free extract," opinion as to the starch content of a great many starch-bearing plant organs has apparently undergone little change in the intervening century.

Existing methods for the determination of starch are based upon a decidedly restricted number of its properties, and those that have been made use of analytically have been known for a great many years. In the analysis of complex materials such as whole wheat, all of the various types of methods appear to have objectionable features or weaknesses. According to Terrier (35), 112 papers have appeared on the determination of starch in the milling products of wheat. Collaborative studies of the A.O.A.C. diastase–hydrochloric (22) and gravimetric procedures (3, 26-28, 30) have revealed large and unexplained interlaboratory differences. A similar lack of agreement between the results reported by different analysts has characterized collaborative studies of the calcium chloride polarimetric procedure (11, 12, 17, 18, 27). While non-existence of dependable reference methods for the determination of starch is now generally acknowledged, reports of comparative applications of assorted methods to complex materials of unknown starch content constitute much of the literature. Since the true starch content of the material analysed is really not known before or after such comparisons are made, the accuracy of the methods studied remains unsettled.

The starch content of whole wheat cannot be determined satisfactorily by difference because of the doubtful accuracy of existing analytical methods for non-starchy solids. Terrier (35) attempted to use this approach but pointed out that starch data obtained in this way are subject to large errors. Quantitative separation of the component fractions followed by reconstitution to provide material of accurately known starch content is not regarded as an acceptable method of providing reference samples. There is yet no report of this ever having been accomplished with such a complex material as whole wheat. Even if it were, the reconstituted product could hardly be claimed to represent the original raw material because of the physical changes that some of the constituents undergo in the course of their isolation. There is little reason for supposing that interfering materials always have the same effect when different samples of the same plant organ are analysed by the

same procedure. It does not necessarily follow that because some one method has been shown to be satisfactory in every way for mature wheat it is equally satisfactory for immature or sprouted grain, or for other types of grain, or that adulterants are without effect, or that the previous history of the sample may be entirely neglected. These circumstances are partially responsible for the unsatisfactory status of methods for the direct determination of starch in complex products.

Progress with polarimetric methods has been anything but spectacular. Fifty years elapsed between the discovery of the high optical rotatory power of starch solutions by Biot and Persoz (5) and Payen (31), and the appearance of the polarimetric procedures of Dubrunfaut and Effront. It was at a much later period that polarimetric procedures that have been found at all suitable for analytical purposes were devised (13, 24). Attention has shifted to some extent from the use of strongly acid or alkaline solutions to concentrated salt solutions as starch solvents, the appearance of Hopkins' procedure (16) being chiefly responsible for this trend. The present tendency also is to avoid the use of protein precipitants capable of precipitating starch. The simplicity of the earlier polarimetric procedures has been lost in part by the addition of extra steps by later workers. The methods of Lintner (24) and Ewers (13) for instance consist in bringing the starch into solution, and in removing soluble proteins by precipitation, after which the extract is brought to volume, mixed, filtered, and polarized. This advantage of simplicity should not be judged merely in terms of diminished labour requirement. Additional steps may introduce unsuspected systematic errors and accentuate the influence of the personal factor in routine applications. Although nearly one hundred papers dealing with the polarimetric determination of starch have already appeared in the literature, no mention is made of this type of starch analysis in the fifth and earlier editions of the *A.O.A.C. Official and Tentative Methods of Analysis* (2) or in the fourth and earlier editions of *Cereal Laboratory Methods* (1). The most recent edition of *Physical and Chemical Methods of Sugar Analysis* by Browne and Zerban (6) includes descriptions of a few polarimetric methods for the determination of starch, but the authors do not consider any of these to be as reliable as the tedious hydrolytic methods also described, at least in the examination of complex products.

Methods based on polarization are unique among macro-procedures in that they offer the possibility of making this analysis about as simple and easy to perform as moisture, ash, or Kjeldahl nitrogen estimations. This explains the continued interest that has been taken in them, despite the objectionable features and questionable reliability of all the well known procedures of this type. Concentrated salt solutions appeared to have attractive features as starch solvents. This type of polarimetric procedure has been studied intensively by the author, with the object of providing methods suitable for reference as well as routine applications.

The Calcium Chloride Polarimetric Procedure

Preliminary

In the course of the analysis, difficulties that were perhaps superficial but none the less serious were encountered, and these had to be overcome before studies of basic problems were undertaken.

Berzelius beakers (400 cc.) are much more suitable as boiling vessels than the small Erlenmeyer flasks (16, 21) and casseroles (25) employed previously. A watchglass cover is unnecessary and is a nuisance when stirring, rubbing down the sides, and adding water to the boiling salt solution; it also increases the frothing tendency considerably and does not eliminate evaporation losses.

If the solid particles are allowed to form gummy aggregates in the preliminary stages of the extraction, precise directions as to particle size obviously are of little real value. The particles must be separated before the starch solvent renders them sticky. The best treatment at this point varies to some extent with the nature of the material. With gluten, for instance, it is preferable to suspend the sample directly in the calcium chloride solution without preliminary wetting (7). With materials of high starch content, a uniform aqueous slurry should first be prepared. Commercial starch samples settle out rapidly to form a compact sediment which, if not redispersed before adding the calcium chloride solution, will lead to quite as much difficulty with lumping as when the preparatory treatment with water is omitted. Properly prepared suspensions will not char over a strong heat source even when little attention is given to stirring.

Many calcium chloride extracts show a pronounced tendency to froth, particularly during the first few minutes' heating. This inevitably leads the analyst to adjust or remove the heat source, with consequent lack of uniformity in the conditions of the boiling treatment. Mild heating also gives rise to filtration difficulties that would not otherwise be encountered. The addition of a few drops of *n*-octanol controls frothing very satisfactorily and is without effect on the polarization value. Since *n*-octanol is removed progressively by steam distillation, only a trace is ordinarily present at the end of the extraction. Large amounts should not be added, particularly toward the end of boiling treatment, because of the tendency of this liquid to form cloudy emulsions when shaken up with the cold extract. Cetyl alcohol added in similarly small quantities has been found useful for the same purpose.

If evaporation losses are not corrected during boiling, the starch is subjected to conditions that change continuously in the course of individual extractions. In the presence of *n*-octanol, maintenance of uniform conditions during boiling is readily effected by the addition of water either from a pipette or constant drip device. Refluxing, autoclaving, and digestion in baths maintained at various temperatures have been tested and are judged less suitable for practical work than boiling in open beakers over heaters (extraction temperature, 109° ± 1° C.; water loss, 1 to 2 cc. per min.).

Slow filtration has often been encountered in earlier studies of this method (27). The relationship of this difficulty to inadequate heating has already been indicated. It is also encountered when the pH is too high, i.e., 3.0 and higher. Cooked products give greater difficulty in this respect than raw flours. Smaller samples and longer boiling periods increase the filtration rate with these materials but the employment of samples of less than 2 gm. is not considered advisable because of the greater importance that observational errors then assume. The filtration is most conveniently carried out at atmospheric pressure.

Cloudy filtrates may arise from the starch itself but accompanying fat is much more apt to be responsible. It is the latter, for instance, that is responsible for the turbidity that develops upon addition of water to cold cereal starch solutions. The cloudiness that develops on adding stannic chloride to cereal starch solutions also arises from dispersed fatty material. Elimination of this difficulty was necessarily antecedent to precise experimental studies of the present method.

Fundamental Studies

Influences of such factors as salt concentration, extraction temperature and extraction time, pH, starch concentration, filtration technique, and polarization temperature on the specific rotation value of starch demanded close scrutiny. Experimental study of these has revealed the need for further standardization of the calcium chloride polarimetric technique (8) and has also provided an explanatory basis for the interlaboratory differences that had previously characterized collaborative applications of this method to commercial starches. Although the A.O.A.C. diastatic and gravimetric procedures have been found anything but superior to the Hopkins method in this respect, a similar demonstration of the cause of interlaboratory differences with these more lengthy methods and of how they may be overcome has yet to appear.

The influence of variety and growth environment on the specific rotation value for particular types of starch has remained uncertain, and, as a result, the reliability of polarimetric methods has remained questionable. A recent comparative study (9) has shown that the specific rotatory power of grain starches is remarkably uniform under the conditions of the improved calcium chloride procedure. The values for grain sorghum starch from Lubbock, Texas, for wheat starches from various stations across Canada, including Beaverlodge, Alta. (Peace River district) and Ottawa, Ont., as well as for starches prepared from various other grains, all showed sufficiently close agreement (9) to justify the adoption of an average figure of 203°. Among the many types of starch included in this study, only the legume and potato starches showed an appreciable divergence from this figure.

Accompanying non-starchy solid constituents influence the optical rotatory power of extracts prepared from complex plant products. Experimental studies of this aspect of the calcium chloride polarimetric procedure with

reference to whole wheat and wheat flours are reported in the present paper. By taking all fractions into consideration, it has been possible to demonstrate the degree of accuracy obtainable with this method. The soluble protein error is overcome satisfactorily by protein precipitants, the use of which reduces the labour requirement very considerably. A feature of the present work has been the demonstration of the influence of hemicelluloses of the bran and endosperm which are dissolved along with the starch.

Recommended Procedure for Whole, Granular, and Patent Wheat Flours

Reagents

(i) Aqueous calcium chloride

Dissolve crystalline calcium chloride ($CaCl_2.6H_2O$ or $CaCl_2.2H_2O$) in distilled water and adjust to a density of 1.30 at 20° C. Filter with suction through several layers of paper until crystal clear. Adjust to a pH of 5.5 with dilute sodium hydroxide and acetic acid. Alternatively the solution may be adjusted *en masse* to pH 2.2 to 2.5 with acetic acid, making it unnecessary to employ Reagent (ii).

(ii) 0.8% Acetic acid

(iii) *n*-Octanol or cetyl alcohol

(iv) 4% Stannic chloride
Dissolve 4.0 gm. of $SnCl_4.5H_2O$ in 100 cc. of Reagent (i)

(v) 5% Uranyl acetate
Dissolve 5 gm. of $UO_2(C_2H_3O_2)_2.2H_2O$ in 100 cc. of Reagent (i)

(vi) 95% Ethanol

Procedure

Whole wheat should be ground to pass a 40 mesh screen*; granular and patent flours are analysed as received. Weigh out 2.463 gm. samples and transfer to 400 cc. Berzelius beakers. Add 10 cc. of distilled water and prepare a smooth suspension with aid of a rubber-tipped glass rod. Add 60 cc. of calcium chloride solution and 2 cc. of 0.8% acetic acid with stirring. Mark level of beakers' contents with a wax pencil. Bring to boiling in four to five minutes with frequent stirring, avoiding overheating the sides. Allow boiling to proceed fairly briskly for 15 min.*, taking care to stir the mixture periodically (rate of water loss, 1 to 2 cc. per min.). Add water during the course of the extraction to ensure uniformity in salt concentration and extraction temperature. If frothing becomes troublesome, add a few drops of *n*-octanol or a small piece of cetyl alcohol. After fifteen minutes' boiling, cool to room temperature. Add 2.5 to 5 cc. of 4% stannic chloride or 10 cc. of 5% uranyl acetate with stirring. Transfer to 100 cc. volumetric flask, rinsing out carefully with successive lots of Reagent (i). Foam in the neck of the flask is destroyed by adding a drop or two of 95% ethanol, shaking as the alcohol

* *The particle size and extraction time requirements will be given detailed consideration in a later publication.*

reaches the froth. Bring accurately to volume with Reagent (i), stopper the flask and mix thoroughly. Filter through 15 cm. Whatman No. 12 fluted paper. Discard the first 20 cc. and collect 30 to 50 cc. for polarization. If filtration is slow, cover the funnels. Polarize in 2 dm. tubes, employing sodium light. % Starch = polarimeter reading in degrees × 10.

Employing 10 cc. of 5% uranyl acetate solution as protein precipitant, the above procedure may also be used for the rapid determination of starch in corn, barley, rye, rice, grain sorghum, and buckwheat.

Experimental

Soluble Proteins

Mannich and Lenz (25) pointed out that levorotatory wheat proteins are dissolved in sufficient amount during fifteen minutes' hot extraction to reduce the apparent starch content of flours by 1 to 2%. It might be argued that variation of this error from sample to sample is not sufficiently large as to necessitate more than the use of a small correction factor. Disposal of the soluble protein effect in this way actually would be preferable to either of the two methods proposed by Mannich and Lenz for its elimination.

Hopkins (16) has demonstrated serious weaknesses in their "by-difference" method (25): much less protein is dissolved in cold as opposed to boiling calcium chloride; sucrose is dextrorotatory under the first and levorotatory under the second set of conditions; and starch is dissolved to some extent by concentrated calcium chloride solution even at 10° C. Hopkins found that preparatory extraction of wheat flour samples with aqueous ethanol (d. 0.88) increased the polarization value of the calcium chloride extract very considerably. This increase was far higher than could be explained by the removal of non-protein interfering substances, thus providing the first evidence that the gliadin fraction otherwise dissolves along with the starch. Hopkins and Graham (19) have also shown that the levorotatory power of the alcoholic extract bears a fairly close relationship to the total protein content of wheat samples. Granting that Hopkins' preparatory extraction eliminates interference by alcohol-soluble constituents, existing evidence concerning proteins of the wheat berry as a whole is not entirely satisfactory. Hopkins' procedure was applied successfully to starch-gluten mixtures (16), the proportions of which corresponded to soft and hard wheat flours. Beyond causing a very slight overestimation of starch content, the presence of gluten was found to have no effect upon the analysis (16). This work left the following questions unanswered: If the isolated gluten had been extracted with boiling salt solution by itself, would the filtered extract have given a positive test for starch with iodine? To what extent had its solubility properties been altered in the course of its isolation? From this study of at least partially denatured gluten, may it be assumed that preparatory extraction with aqueous ethanol eliminates errors arising from the proteins of the entire kernel? Further work using a different approach seemed desirable.

Filtrates prepared according to Hopkins' directions from the whole wheat samples described in Table II gave negative tests for protein on addition of an excess of stannic chloride, as well as on saturation with picric acid. Kjeldahl analyses on 25 cc. aliquots showed filtrate nitrogen contents of 3 to 4.5 mgm. per 100 cc. for patent flours and 7.5 to 10.0 mgm. per 100 cc. for whole wheat, employing 2.5 gm. samples. The protein content of the sample was not reflected in the nitrogen content of the filtered calcium chloride extract. In applications to whole wheat or patent flours, Hopkins' preparatory extraction evidently eliminates the soluble protein error entirely.

Following this preparatory treatment, calcium chloride extracts of the residue are always slightly turbid, making it impossible to take accurate readings on 2 dm. tubes. Clearer filtrates are obtained when Hopkins' pretreatment is followed by extraction with absolute ethanol and ethyl ether, the "alcohol–ether" data of Table II being obtained in this manner.

Mannich and Lenz suggested precipitation with an excess of stannic chloride as a means of removing soluble wheat proteins from calcium chloride extracts (25). They stated that this reagent had no effect on the optical rotatory power of wheat starch solutions.

When stannic chloride (dissolved in stock calcium chloride solution) was added to wheat-starch–calcium-chloride solutions, dense cloudiness developed at once although no floc was formed. This did not occur with root and tuber starches, and with these a pronounced decrease in the polarization value was observed. Since associated fats appeared to be responsible for the cloudiness encountered with cereal starches, wheat and corn starch were defatted by 72 hours' extraction with 80% dioxane as directed by Schoch (33). Residual solvent was removed with 85% methanol and the samples were then dried in an air tunnel. This treatment reduced the fat content to 0.1% and was without effect on the specific rotation value expressed under carefully standardized conditions. After this defatting treatment cloudiness did not develop on adding stannic chloride to the cereal-starch–calcium-chloride solution.

Fig. 1 shows that an excess of stannic chloride leads to a remarkable decrease in the specific rotation value of wheat starch. This effect is particularly pronounced at low concentrations of stannic chloride, becoming proportionately less in the presence of larger amounts. A similar depression of the polarization value was observed with potato, waxy corn, and defatted corn starch (Table I). The concordance observed between ordinary and waxy corn starch indicates that stannic chloride has a similar effect on the amylose and amylopectin components. The decrease in polarization value is observed immediately after the addition of stannic chloride, the value then remaining unchanged on standing for many hours. The effect of stannic chloride, therefore, cannot be attributed to hydrolysis. Evidently it becomes closely associated with the dispersed starch, its presence leading to a decrease in the optical rotatory power of immediately adjacent carbon atoms.

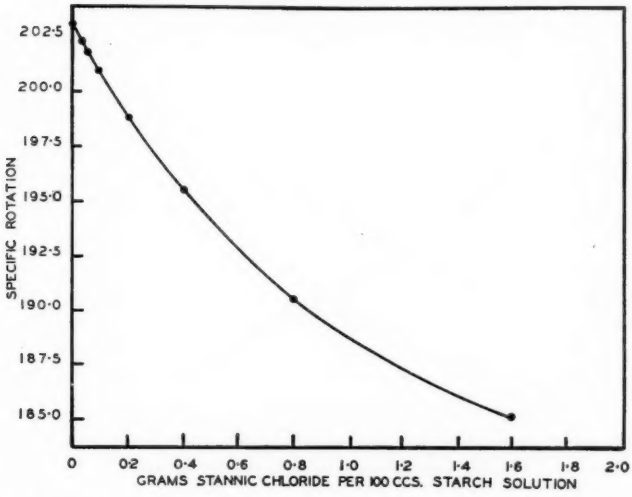

FIG. 1. *Effect of increasing amounts of stannic chloride on the specific rotatory power of defatted wheat starch.*

TABLE I

EFFECT OF STANNIC CHLORIDE ON THE POLARIZATION VALUE OF DEFATTED CORN, WAXY CORN, AND POTATO STARCHES

—	2 dm. polarization value, degrees		
	Defatted corn	Waxy corn	Potato
Control	7.15	7.21	7.08
Treated*	6.65	6.68	6.43

** 5 cc. of 20% stannic chloride added before making to volume.*

Figs. 2, 3, and 4 show the effects of increasing amounts of stannic chloride on the optical rotatory power and nitrogen content of filtrates prepared from 2.5 gm. (100 mesh) samples of whole wheat and patent flours. All filtrates could be polarized without difficulty in 2 dm. tubes, the readings on duplicate extracts agreeing within 0.01° to 0.02°. Addition of 0.1 to 0.2 gm. of $SnCl_4.5H_2O$ (2.5 to 5.0 cc. of 4% solution) increased the polarization value to approximately that observed with preparatory alcoholic extraction, the nitrogen content of the filtrate being drastically reduced. When greater amounts of stannic chloride were employed, the nitrogen content was reduced still further but the polarization value was then depressed very considerably, as might be expected from Fig. 1. The addition of 5 cc. of 20% stannic chloride solution as recommended by Mannich and Lenz (25) actually led to lower polarization values than when deproteinization treatments were entirely omitted.

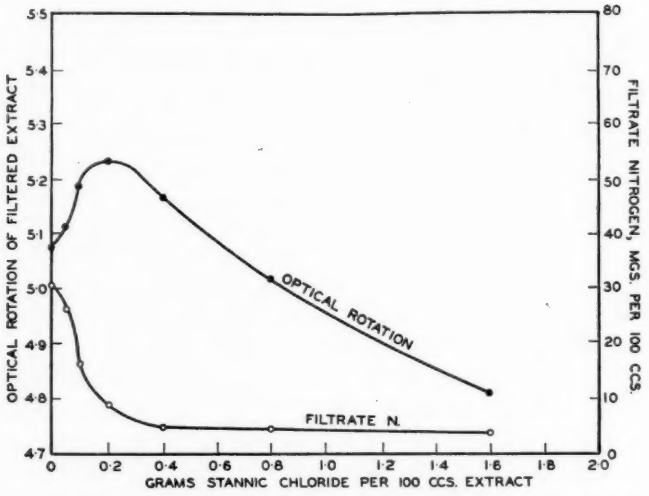

FIG. 2. *Effect of increasing amounts of stannic chloride on the optical rotatory power and nitrogen content of whole wheat filtrates (high protein).*

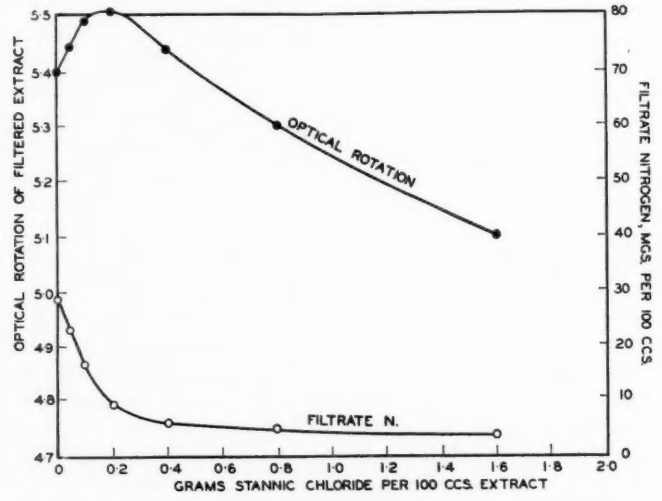

FIG. 3. *Effect of increasing amounts of stannic chloride on the optical rotatory power and nitrogen content of whole wheat filtrates (low protein).*

From these observations, it is evident that stannic chloride must be used with great care in the polarimetric determination of starch. The decrease observed with excessive amounts suggests that even with 0.1 to 0.2 gm., the optical rotatory power of the starch is depressed to some extent. While comparisons with alcohol–ether pretreatment (Table II) indicated that this

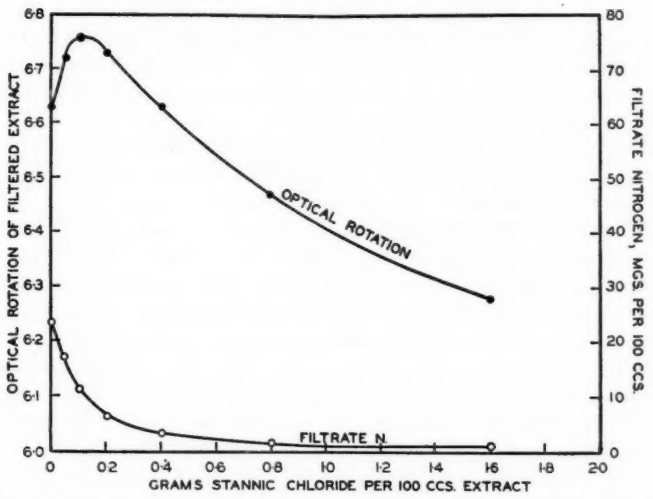

FIG. 4. *Effect of increasing amounts of stannic chloride on the optical rotatory power and nitrogen content of patent flour filtrates.*

TABLE II

COMPARISON OF DEPROTEINIZATION METHODS

Sample	Protein, %	% starch, dry basis, assuming $\alpha = 203°$		
		Alcohol–ether	2.5 cc. of 4% stannic chloride	5.0 cc. of 4% stannic chloride
Whole wheat	19.9	57.2 57.4	57.2 57.2	57.1 57.2
Whole wheat	19.6	59.7 59.6	59.3 59.3	59.7 59.5
Whole wheat	17.1	59.9 59.8	60.0 60.0	60.0 60.0
Whole wheat	16.0	63.2 63.2	63.1 63.1	63.1 63.0
Whole wheat	15.2	62.1 62.1	62.0 61.9	62.0 62.0
Whole wheat	14.8	62.4 62.3	62.0 62.2	62.2 62.4
Whole wheat	13.3	65.1 65.2	65.0 65.0	65.0 64.8
Whole wheat	11.8	65.0 65.0	64.9 64.9	64.8 64.8
Hard patent flour	13.9	78.1 78.1	77.5 77.5	77.6 77.7
Soft (cake) flour	10.4	82.0 82.0	81.6 81.6	81.5 81.5

error is a very small one, confirmatory evidence based on estimations of residual stannic chloride appeared desirable. The sensitivity of calcium chloride extracts of gluten to low concentrations of stannic chloride as evidenced by incipient flocculation provided a suitable basis for a semi-quantitative test. For this purpose, the extract obtained by boiling 4 gm. of 100 mesh gluten (air-dried) for 30 min. with 100 cc. of calcium chloride solution was found suitable. Upon mixing small volumes of the resulting filtrate 1 : 1 with calcium chloride solution of known stannic chloride content, cloudiness appeared at 0.03% and a definite precipitate at 0.06% of stannic chloride (as $SnCl_4.5H_2O$). Stannic-chloride–treated extracts of whole wheat and patent flour were then mixed with the gluten extract and matched with standards. When 2.5 cc. of 4% stannic chloride had been employed, the residual stannic chloride concentration was found to be slightly less than 0.05%, whereas with 5.0 cc. it amounted to approximately 0.1%, i.e., half of the added precipitant was removed with the protein at the latter level. Little difference could be detected between different flours in this respect. From Fig. 1 it is evident that a stannic chloride concentration of 0.1% reduces the polarization value for starch by 1%. Barring compensatory effects, this signifies that an error amounting to as much as 0.6% starch may result from its use in the analysis of whole wheat.

Table II reports comparative applications of the calcium chloride polarimetric procedure with protein removal (*a*) by alcohol–ether extraction; (*b*) by precipitation with 2.5 and 5.0 cc. of 4% stannic chloride. In carrying out these analyses, cognizance was taken of the factors dealt with in an earlier paper (8), the conditions of the analysis differing only with respect to the deproteinization treatment. The 80 to 100 mesh whole wheat samples represented soft, hard, and durum varieties, and differed considerably in protein content. All extracts filtered satisfactorily and the filtrates were clear enough to permit the use of 2 dm. tubes. With all three methods, the results were in satisfactory agreement irrespective of the protein content of the sample. In view of this concordance it is evident that the composition of the precipitate varies to some extent with the protein content of the sample. While the stannic chloride values tended to be slightly lower, particularly on flours, in no instance did the discrepancy between methods exceed 0.6% of starch.

Steiner and Guthrie (34) have recently suggested the use of aqueous uranyl acetate solution as a protein precipitant in a calcium chloride polarimetric procedure for sweet potatoes. The author has found that this compound may be used to good advantage in the polarimetric determination of starch in cereals and other grains. It should be dissolved in stock calcium chloride solution in order to avoid cloudiness; water added by itself to cold cereal-starch–calcium-chloride solutions causes cloudiness, and the same is very apt to be encountered when relatively dilute aqueous solutions are used to precipitate soluble proteins.

Table III shows that the optical rotatory power of wheat starch solution remains unchanged when 10 to 30 cc. of 5% uranyl acetate (Reagent (v)) is

TABLE III

EFFECT OF DIFFERENT VOLUMES OF 5% URANYL ACETATE SOLUTIONS ON THE POLARIZATION VALUE OF WHEAT-STARCH–CALCIUM-CHLORIDE SOLUTIONS

Ccs. added	Polarization value, 2 dm.	
	5% uranyl acetate dissolved in water	5% uranyl acetate dissolved in stock calcium chloride solution
0	7.43	7.43
10	7.42	7.43
20	7.41	7.44
30	7.39	7.42

added prior to volume adjustment. The polarization value is depressed by the addition of uranyl acetate dissolved in water since the calcium chloride concentration is then reduced.

From Table IV it appears that 10 cc. of 5% uranyl acetate is adequate for 2.5 gm. wheat flour samples, larger amounts causing no further increase in the polarization value. Using this precipitation treatment, the starch values

TABLE IV

EFFECT OF DIFFERENT VOLUMES OF 5% URANYL ACETATE (DISSOLVED IN AQUEOUS CALCIUM CHLORIDE) ON THE OPTICAL ROTATORY POWER OF WHEAT FLOUR EXTRACTS

Sample	Protein content, %, dry basis	Polarization value, 2 dm.			
		Volume of 5% uranyl acetate added, cc.			
		5	10	15	20
Whole wheat	19.22	5.11	5.17	5.17	5.17
Whole wheat	15.16	5.67	5.73	5.73	5.72
Whole wheat	13.33	5.89	5.92	5.93	5.93
Whole wheat (durum)	16.01	5.73	5.75	5.76	5.76
Soft (biscuit) flour	10.35	7.25	7.29	7.28	7.28
Hard patent flour	13.91	6.85	6.91	6.91	6.92

for soft and hard patent flours coincided with those observed with alcohol–ether extraction (Table II); the apparent starch contents of four whole wheat samples (protein content, 12 to 19%) averaged 0.2% higher, the differences ranging from 0 to 0.6%.

Employing the alkaline wash recommended by Earle and Milner (10), filtration of the calcium chloride extract was faster and the filtrates tended to be slightly clearer than usual. Starch measurements obtained with this method on patent and whole wheat flours averaged the same, however, as when Hopkins' pretreatment was employed, the differences observed on particular samples never amounting to more than 0.2% of starch.

Hemicelluloses

Boiling calcium chloride solution has previously been assumed to act as a selective solvent for starch, the hemicelluloses of the endosperm and bran remaining insoluble. The literature reveals no evidence in support of this assumption, however, beyond polarimetric observations made on calcium chloride extracts of wheat straw (16, 25).

Direct analysis of filtered calcium chloride extracts of whole wheat and patent flour has shown that furfural-yielding substances are present in considerable amount (Table V). In carrying out these determinations, 50 cc.

TABLE V

PENTOSAN CONTENT OF CALCIUM CHLORIDE EXTRACTS OF WHEAT FLOURS

Sample	Flour pentosan content, %, dry basis	Furfural yield, mgm./100 cc. calcium chloride filtrate	Pentosan content of filtrate, mgm./100 cc.	Pentosans dissolved, %, dry basis
Whole wheat	7.25	28.7	47.6	2.14
Whole wheat	6.65	26.6	44.2	2.01
Hard patent	2.10	12.4	20.5	0.93

of calcium chloride filtrate and 50 cc. of 24% hydrochloric acid were mixed and then subjected to the standard distillation procedure for pentosans (2), 12% hydrochloric acid being introduced at the usual rate. The distillation was continued until no further furfural was carried over, as judged by qualitative tests with aniline acetate. The distillate was redistilled from saturated sodium chloride as directed by Schmidt–Nielson and Hammer (32). Furfural was determined by the bromination procedure of Hughes and Acree (20). Since blank determinations on starch–calcium-chloride solution showed that redistillation does not remove hydroxymethylfurfural entirely, this source of error was taken into consideration in assessing the probable amount of hemicellulose that is dispersed.

Table V shows that as much as 1% pentosan may be dissolved along with starch in applying the calcium chloride polarimetric procedure to patent wheat flours. Approximately double this amount is dissolved in the case of whole wheat flours, while with commercial wheat bran, the amount dispersed is vastly greater (Table VII). Preparatory extraction with alcohol–ether and protein precipitation treatments were found to have no effect on the furfural yield of calcium chloride extracts. While preparatory extraction of the flour samples with 0.05 *N* sodium hydroxide (10) reduced the furfural yield by approximately 25%, this treatment did not exclude soluble hemicelluloses; the faster filtration that characterizes the Earle–Milner procedure is to be attributed to the removal of salt-insoluble proteins and reduced sample size as well as to partial removal of hemicelluloses.

In view of their solubility, information was sought on the specific rotatory power of endosperm and bran hemicelluloses under the conditions of the calcium chloride polarimetric method for starch.

Water-soluble pentosan was isolated from patent wheat flours essentially by the procedure described by Baker, Parker, and Mize (4). Ice water was employed in preparing the batters. After two precipitation treatments with cupric hydroxide, the pentosan remaining in solution was salted out with ammonium sulphate. The gum was dissolved in water and dialyzed in Visking sausage casing against distilled water for 48 hr. The pentosan was recovered by pouring into two volumes of 95% ethanol. The precipitate was dried with increasing strengths of ethyl alcohol followed by ethyl ether. The yield from 400 gm. of flour amounted to 1.18 gm.; moisture (100° C. *in vacuo* to constancy), 6.3%; protein (N × 5.7), 1.6%; ash, 0.7%; reducing sugars (as dextrose), by acid hydrolysis, 105.7%; furfural yield, 60%; pentosan content assuming 83% theoretical yield of furfural, 99.34% (air-dry basis). Qualitative tests for protein (Biuret, Millons) were negative and the Lassaigne test for organic nitrogen was only faintly positive. Iodine tests for starch were negative on the dry powder as well as on its aqueous solution.

The endosperm pentosan dissolved readily in concentrated calcium chloride solution. Employing 0.2 gm. samples, a specific rotation value of − 25° to − 30° was observed under the conditions of the polarimetric procedure. The effect of this wheat constituent on the polarimetric measurement is thus of a very low order of magnitude; assuming that patent wheat flour contains 1%, the soluble pentosan error amounts to less than 0.2% of starch. This gum also accounts for nearly half of the pentosan dissolved in the analysis of whole wheat.

Since there was clear evidence of soluble bran pentosans, an effort was made to determine their effect upon the polarimetric analysis as well. Microscopic examination of commercial wheat bran showed that the cell wall material does not stain blue with iodine. Hemicellulose fractions *A*, *B*, and *C*, prepared according to the directions of Norris and Preece (29), stained blue with iodine, however, and the yields were low. The following procedure provided a starch-free product of low protein content. Wheat bran (200 gm., 100 mesh) was digested successively with malt extract under conditions similar to those of the A.O.A.C. diastase method for starch. The residue was recovered by centrifuging, the extract being discarded. The solids were then dispersed uniformly in two litres of water. After adjusting to pH 8.0 with phosphate buffer, 2 gm. of trypsin was added in aqueous solution. The suspension was maintained at 40° C. for 12 hr. with continuous stirring. The insoluble solids were recovered by centrifuging and were washed thoroughly with distilled water. A uniform suspension was then prepared in two litres of 4% sodium hydroxide. After stirring mechanically for two hours at 25° C., the extract was recovered by centrifuging. The extraction was repeated with a further two litres of 4% sodium hydroxide. The combined

extract was clarified with diatomaceous earth. The clear amber liquid was neutralized with acetic acid to pH 4.0, this having been found most suitable by previous experiment. The extract was then made 55% with respect to ethanol. The heavy precipitate was collected by centrifuging. After careful washing with 60% ethanol it was dried with alcohol and acetone. The yield of air-dry material was 17.8 gm.; moisture, 5.84%; protein, 2.6%; ash, 6.33%; reducing sugars by acid hydrolysis, 85.60%; pentosan (redistilled), 70.6%; same on dry ash-free basis, 80.0%.

When dissolved in 100 cc. of 2% sodium hydroxide, 0.5 gm. of the air-dry sample gave a 2 dm. polarization value of $-0.30°$. The same polarization value was observed under the conditions of the present method for starch but in this instance a small part of the sample, presumably lignin, remained insoluble. The specific rotation for this mixture of cell-wall substances, corrected for moisture, ash, and protein, is taken to be $-35°$. In the routine analysis of whole wheat, between 1.0 and 1.3% of salt-soluble bran pentosan is apparently dispersed. Taking the upper figure, an error of -0.3% (as starch) may arise from this source. In the analysis of whole wheat, the combined error of underestimation arising from furfural-yielding constituents of endosperm and bran is not greater than 0.5%, expressed again as starch. The magnitude of this error should be far higher in the polarimetric determination of starch in wheat bran (Table VII); here the dispersed hemicelluloses should reduce the apparent starch content by 1.5 to 2.0%.

In applications of the present polarimetric procedure to patent and whole wheat flours, the effect of soluble hemicelluloses is small by reason of their low specific rotatory power, and towards underestimation because of their levorotatory nature. The accuracy of the method in their presence rests on these considerations, not on the selectivity of hot calcium chloride solution as a starch solvent.

No attempt has been made to determine the actual quantity of furfural-yielding substances that are brought into solution along with starch in applications of the diastase–hydrochloric (2) and Rask procedures (2) to wheat products. Omitting preparatory washing, the starch-free hemicellulose fractions isolated from endosperm and bran were subjected to these two procedures. A high "starch" content was indicated.

	Apparent starch content, %, air-dry basis	
	Diastase–hydrochloric	Rask
Endosperm pentosan	97.3	68.0
Bran hemicellulose	49.9	17.4

The hemicellulose components of wheat may cause overestimation of starch content by either of these methods, the diastase method apparently being more apt to suffer from this defect than the Rask.

Insoluble Solids

The effect of insoluble solids other than precipitated proteins has been determined by extracting whole wheat and patent flour samples (2.5 gm.)

successively with hot calcium chloride solution, with filtration in steam jacketed funnels, extraction being continued until the calcium chloride filtrates were starch-free. Subjection of the residue to the conditions of the polarimetric procedure provided filtrates possessing no optical rotatory power, so that soluble proteins and starch did not interfere in the experiments that followed. Wheat-starch–calcium-chloride solutions were then prepared in the presence and absence of the insoluble residues. The polarization value for the starch by itself was + 7.145°. In the presence of whole wheat residue, the value was increased to 7.20–7.21° (five experiments). With patent flour residue, the polarization value was 7.17° (two experiments). These results show that the presence of insoluble solids leads to a greater error of overestimation than would be expected from the weight and density of this material. Evidently this error arises from two separate effects; (*a*) liquid displacement, (*b*) selective sorption of starch solvent or water. With whole wheat, this error of overestimation amounts to 0.8% of the true polarization value, or approximately 0.5% starch. With patent flour, overestimation from this cause amounts to 0.35% of the polarization value, or 0.2 to 0.3% of starch. Evidently the small errors arising from levorotatory hemicelluloses in the analysis of whole wheat and patent flour are balanced by the opposing effect of insoluble solids.

Non-protein Alcohol Extractives

The influence of substances other than proteins that are removed by preparatory alcoholic extraction has been determined by adding the alcohol extract to the usual volume of calcium chloride solution, boiling off the alcohol and surplus water, and continuing the boiling treatment for a further 15 min. at 109° C. On cooling, 5 cc. of 4% stannic chloride was added. The 2 dm. polarization value for protein-free filtrates from 2.5 gm. samples of whole wheat ranged from − 0.045 to − 0.055°, which amounts to 0.5% of starch. The corresponding values for granular and patent flours were − 0.03° or 0.3% of starch. These values were confirmed by determinations on 10 gm. samples. Prolonged refluxing with 65% ethanol did not remove additional optically active substances. The total sugar content of the samples employed was approximately 4.0% and 2.0%, respectively, for the whole and patent flour samples.

Fat

Ether-soluble constituents are capable of causing cloudiness but no measurable influence on the polarization value has been found in experiments similar to the foregoing.

Mineral Constituents

On mixing the ash obtained by incineration of 4 gm. of wheat samples with wheat starch prior to starch dispersal, a small increase in the polarization value was observed. This evidently resulted from the volume occupied by insoluble ash since calcium chloride extracts of the ash upon filtration had no effect on the starch polarization value.

Variation in Solubility of Proteins and Hemicelluloses with Salt Concentration

Wheat starch has already been shown to be completely soluble in hot calcium chloride solutions of widely different densities (8). Table VI shows that gluten solubility drops off rather sharply with increasing salt concentration. This was confirmed by the addition of solid calcium chloride to filtered

TABLE VI

SOLUBILITY OF GLUTEN PROTEINS IN CALCIUM CHLORIDE SOLUTIONS OF DIFFERENT CONCENTRATIONS

Calcium chloride solution density	Optical rotation (2 dm.) before protein removal	Optical rotation (2 dm.) after protein removal	Difference
1.25	− 0.57°	+ 0.44°	1.01°
	− 0.54°	+ 0.44°	0.98°
1.35	− 0.43°	+ 0.44°	0.87°
1.43	+ 0.11°	+ 0.43°	0.32°
	+ 0.05°	+ 0.43°	0.38°

gluten extracts prepared with relatively dilute salt solution (d. 1.25), considerable protein then being rendered insoluble. Increasing the salt content of the boiling solvent promotes the solubilization of bran hemicellulose, the furfural yield of bran extracts being almost doubled when the solvent density was increased from 1.25 to 1.43 (Table VII). The solubility of proteinaceous

TABLE VII

SOLUBILITY OF BRAN PENTOSANS IN CALCIUM CHLORIDE SOLUTIONS OF DIFFERENT CONCENTRATIONS

Calcium chloride solution density	Furfural yield, mgm. per 100 cc. filtrate	Pentosan content of filtrate, mgm. per 100 cc.	Pentosans dissolved, gm. per 100 gm. dry bran
1.25	132.0	218.7	6.01
	134.0	222.0	6.09
1.35	193.3	320.2	8.78
	192.5	319.0	8.75
1.43	242.0	403.7	11.07
	241.5	400.0	10.96

and furfural-yielding constituents of wheat would thus appear to be affected differentially by changes in salt concentration. The effects of salt concentration in the present polarimetric analysis are not restricted entirely to changes in the specific rotatory power of starch, but widely different salt concentrations might be used successfully so long as the conditions are the same in the cali-

bration and application of the method. It is quite conceivable that deviation from the customary brine density of 1.30 may prove advantageous in certain applications as a means of restricting the solubilization of non-starchy constituents.

Discussion

The essential requirements of the present analytical procedure are as follows: grinding of the sample to pass a 40 mesh sieve; preparation of a lump-free aqueous suspension with which the acidified starch solvent is then mixed; extraction of the starch by boiling for 15 min. at constant temperature and salt concentration; removal of levorotatory proteins from the cooled extract by precipitation; determination of starch content by polarization of the filtrate. The accuracy of this analysis is considered to be largely a question of the over-all influence of non-starchy solids.

The effects of non-starchy wheat solids might feasibly be corrected for in several ways. The clear calcium chloride filtrate could be polarized directly without recourse to preparatory extraction or protein precipitation treatments, allowance being made for systematic error by use of a correction factor. Such determinations would be open to justifiable criticism since the large error arising from soluble proteins varies considerably from sample to sample. If the soluble proteins are removed by precipitation or with selective solvents, this objection no longer applies. Provided that the lesser influences of the remaining non-starchy components are known and fluctuate little from sample to sample, it should then be possible to calculate the true starch content from polarization measurements.

Hemicelluloses, alcohol-soluble constituents other than proteins, and insoluble solids have been shown to be further fractions requiring consideration. When protein precipitants are employed, it is necessary to consider all three of these, as well as the additional insoluble matter obtained by precipitation, and the influence of residual precipitant upon the optical rotatory power of the dissolved starch. Employing preparatory extraction with aqueous ethanol, the polarization value is influenced only by hemicelluloses and insoluble solids. This also applies to the "alkali-wash" of Earle and Milner (10), except that here the influence of each of these fractions should be lessened.

From the sum of the separately determined influences of non-starchy solids, the systematic error may be estimated for each of the preparatory extraction and precipitation treatments considered in this paper. The effects of levorotatory hemicelluloses is about equal to the overestimation arising from calcium-chloride–insoluble solids. This signifies a negligible systematic error when preparatory extraction with aqueous ethanol (16) or dilute sodium hydroxide (10) is employed, barring errors in technique, and, with respect to the latter (10), loss of starch to the wash liquid. When uranyl acetate is employed, alcohol-soluble non-proteins should reduce the apparent starch content by 0.5% and 0.25% for whole and patent flours, respectively. When stannic chloride is used, the apparent starch content should also be reduced to some

extent by reason of its secondary effect on starch but the total error (as starch) should not exceed 1% with either whole or patent flour. With either precipitant the error of underestimation should be lessened as a result of liquid displacement and sorption by the protein precipitate. All of these refer to calculations based on a specific rotation value of + 203° for starch. When the conventional value of 200° is employed, the starch content would be overestimated slightly, regardless of which deproteinization technique is employed.

Systematic differences have been reported in comparative applications of enzymic, gravimetric, and polarimetric procedures to wheat flours. Hopkins (16) found that results obtained with the calcium chloride polarimetric method were lower than with the Rask procedure, a specific rotation value of + 200° being used in the calculations. Using a higher specific rotation value (+ 203°) Earle and Milner (10) obtained lower results with the calcium chloride polarimetric procedure than with the diastase–hydrochloric-acid method. They found that this difference was diminished by extracting the samples with dilute sodium hydroxide before treatment with malt extract, but on the average it still amounted to 1% of starch. Information presented in the present paper indicates that hemicellulose constituents are at least partially responsible for these discrepancies. Discussion of these small differences might seem premature in view of the interlaboratory differences that have usually characterized collaborative studies of analytical methods for starch. Differences of the same order are involved, however, when a specific rotation value of + 203° is substituted for the lower value used by Mannich and Lenz (25), Jirak (21), and Hopkins (16).

Experiments to be reported subsequently have shown that reducing the particle size and increasing the extraction time does not lead to an increase in the polarization value of whole wheat extracts. Since the present evidence demonstrates that non-starchy solids have a negligible effect on the analysis when preparatory extraction methods are employed, it is concluded that the calcium chloride polarimetric method for starch in wheat is now the most accurate as well as the most convenient of existing procedures. Either protein precipitants or preparatory extraction treatments may be employed in this method of analysis. Use of the former reduces the labour requirement very considerably and for this reason is judged most suitable for routine applications. Preparatory extraction treatments appear most suitable for reference purposes since removal in this way of the greater part of the non-starchy solids simplifies the conditions under which the starch is finally determined.

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A COMPARISON OF FIVE METHODS FOR DETERMINING STARCH CONTENT OF WHEAT[1]

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Abstract

Duplicate determinations made on 17 samples of ground wheat, covering a wide range of starch content, gave mean values and standard errors (for single determinations) for five starch methods as follows: Hopkins, 63.3 ± 0.31; Clendenning, 62.8 ± 0.40; Lintner–Schwarcz, 62.9 ± 0.48; malt diastase with acid hydrolysis, 63.6 ± 0.57; Rask, 62.2 ± 0.48. The standard errors include all analytical errors, as duplicate determinations were made in random order and on different days. The Hopkins method may well be the most accurate as well as the most precise, but the Clendenning method is preferred because of its simplicity and rapidity.

In order to select the most suitable method for routine determination of the starch content of hard red spring wheat, a comparative study has been made of two chemical and three polarimetric starch methods, namely, malt diastase with subsequent acid hydrolysis, the Rask method, the Lintner–Schwarcz method, and the Hopkins and Clendenning modifications of the Mannich–Lenz method. Comparisons were made with wheat samples varying widely in starch content in order to determine whether the relations between results obtained with different methods were constant over the whole range or differed at high and low starch contents.

Materials and Methods

Duplicate determinations were made on 17 samples of wheat. These represented carlots of sound wheat grading as follows: four samples of No. 1 Northern, five of No. 2 Northern, six of No. 3 Northern, and two of No. 2 C. W. Garnet. The correlation between protein and starch contents was used to select the samples on a protein basis from a much larger number so as to obtain a series covering a wide range of starch content with reasonable uniformity. All samples were cleaned free from dockage and were subsequently reduced to an impalpable powder by ball milling.

The official malt–diastase method with subsequent acid hydrolysis was used as described by the Association of Official Agricultural Chemists (2). Dextrose was determined by Allihn's gravimetric procedure; Allihn's table was used for converting the copper oxide obtained to dextrose, and the factor .90 for converting from dextrose to starch.

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The Association accepts the Rask procedure (16) as a tentative method for uncooked cereal products, and the method was used as it is described (2).

The Hopkins (10) method is also accepted by the Association as a tentative method. The only change made in the procedure involved boiling the starch suspension in a 600 ml. beaker (Griffin low form) covered by a clock glass.

Clendenning's (4) modification of the Mannich–Lenz procedure employing stannic chloride was used with the minor variations in procedure reported by Anderson and Eva (1).

The Lintner–Schwarcz method, as described by Ayre, Sallans, and Anderson (3), has been used in this laboratory for both barley and corn. In adapting it for use with wheat it was found that filtration is facilitated by the preliminary washing with ether and alcohol used with the Hopkins method (11). A 2.5 gm. sample is washed once with ether and six times with alcohol (65% by weight). It is then mixed with 50 ml. of sulphuric acid (sp. gr. 1.40) and transferred to a 250 ml. Florence flask, which is placed in a water-bath at 20° C. for one hour and shaken frequently. Subsequently there are added 10 ml. of freshly prepared 2% phosphotungstic acid solution and 40 ml. of sulphuric acid (sp. gr. 1.30). The mixture is well shaken, allowed to stand for 10 min., shaken again, and filtered through a fluted Whatman No. 44 paper. The first 10 ml. is used to wet the paper and is allowed to drain through before proceeding with the main filtration.

All polarimetric readings were made in a 10 cm. tube at room temperature in a Hilger polarimeter with an Osram sodium lamp. A set of 10 readings was made on each test solution, and the Clendenning and Wright (5) figure of 202.7 for the optical rotation of wheat starch was used for converting polarimeter readings to starch. All starch values were computed to a dry basis.

It was found most convenient to make the malt-diastase and Clendenning determinations on the same day, and the Hopkins, Lintner–Schwarcz, and Rask determinations on a second day; two days therefore represented the basic time unit. Duplicate determinations on the 17 samples were allotted in random order to the 17 time units required to complete the work. This procedure provides a very strict test of reproducibility since duplicate determinations are rarely made on the same day.

All determinations were made by one analyst who had previously practised with all five methods.

Results and Discussion

Reproducibility

The standard error of a single determination was calculated for each method from the 17 sets of duplicate values. Results were as follows:

Hopkins	± 0.31
Clendenning	± 0.40
Lintner–Schwarcz	± 0.48
Rask	± 0.48
Malt diastase	± 0.57

These errors are probably higher than would be expected from consideration of the various pairs of duplicate results reported in the literature. But it must be remembered that the duplicate subsamples were analysed on separate days, and often several days apart. This provided a rigorous test of the analytical error. Better checks can doubtless be obtained by making duplicate determinations on the same day and in the same batch, but this does not increase the accuracy of the mean result. Indeed, the practice may give a biased impression of over-all accuracy because it provides no measure of the inter-day laboratory errors.

In our hands, the Hopkins method is the most precise, and is followed by the Clendenning method. The Lintner–Schwarcz and Rask methods are slightly less precise, and the malt–diastase method is definitely the poorest.

Mean Values

The mean starch content of all samples by all methods was 63.0%, and the individual methods gave the following mean values:

Malt diastase	63.6
Hopkins	63.4
Lintner–Schwarcz	62.9
Clendenning	62.8
Rask	62.2

An analysis of variance showed that the significance of the difference between means exceeded the 1% level, and a further analysis, restricted to the three polarimetric methods, showed that the Hopkins value differed significantly (5% level) from the other two.

The malt–diastase method might well be expected to give the highest result and the Rask method the lowest. Malt extract contains enzymes other than amylases, and the method determines all reducing substances formed during hydrolysis, some of which may well arise from compounds other than starch and thus raise the apparent starch content. This source of error may be offset in part by other errors such as that caused by failure to convert all starch to dextrose during the boiling with hydrochloric acid. The Rask method, because it seeks to recover all the starch for weighing, will give low results if any starch is lost, and can give high results only if substances other than starch are retained; the balance of probability favours low results.

Jones (12, 13) found, in agreement with the present study, that the malt–diastase method gives higher results than Rask's method. However, Etheredge (8) reported the same results for the starch content of wheat for both the malt–diastase and the Rask methods. Hopkins (10) and Etheredge (8) found that the Rask method gave higher results for wheat and wheat flour than did Hopkins' own method, whereas Munsey (14) obtained good agreement between the Rask and Hopkins methods for wheat flour. These discrepancies may be explained in part by differences between the samples used by each investigator. In the present investigation, the Rask method

gave higher results than the malt–diastase method for 4 of the 17 samples, and a higher result than the Hopkins method for one sample.

There is some question as to the best factor for converting dextrose to starch in the malt–diastase method. The Association of Official Agricultural Chemists specifies 0.90. But even as far back as 1904, Noyes *et al.* (15) suggested that 100% recovery of the dextrose could not be obtained by acid hydrolysis and recommended the factor of 0.93, and in 1941 Etheredge (7) followed this suggestion in his survey of starch methods. In 1944, Etheredge (9), as referee for the A.O.A.C., gave the opinion that though 0.90 is definitely too low it was best to use it as no other reliable figure was available. Earle and Milner (6), also reporting in 1944, derived the factor 0.92 by experiments with pure dextrose under the conditions used in the malt–diastase method, and with this factor found that the malt–diastase method gave starch values that were 2.5% higher than those obtained by their own modification of the Mannich–Lenz method. If 0.92 had been used in the present investigation, the malt–diastase method would have given a value of 65.0, which is 2% higher than the values given by the polarimetric methods and nearly 3% higher than the value obtained by the Rask method.

It is not possible to say which is the most accurate of the polarimetric methods. The Hopkins method gave a value that was 0.5% higher than values for the other two methods. There is some reason to believe that the Hopkins value may be the most accurate. The argument is simply that if proteins are the main interfering substances, then the method that gives the highest result is most effective in removing proteins and is therefore the best. However, it is probable that there are cancelling errors even with the Hopkins method. In any event, the agreement between the three polarimetric methods is considered good.

Individual Values

Because the Hopkins method is the most precise, and because it may well be the most accurate, it is logical to select it as a standard with which to compare the other methods. The scatter diagrams given in Fig. 1 show the comparative results obtained by the Hopkins method and each of the other four. The correlation with the Hopkins method was good for the two other polarimetric methods, somewhat lower for the malt–diastase method, and much lower for the Rask method. The correlation coefficients for each method compared with each other method are given in Table I. In general the correlations between the polarimetric methods were best, and the malt–diastase method was more highly correlated with these than was the Rask method. The malt–diastase method was best correlated with the Clendenning method, and the Rask with the Lintner–Schwarcz. In view of the fact that its reproducibility is low, the malt–diastase method does comparatively well. On the other hand, the low coefficients given by the Rask method certainly suggest that it is the least satisfactory of all the methods investigated. In other words, if the remaining methods are considered to measure true starch

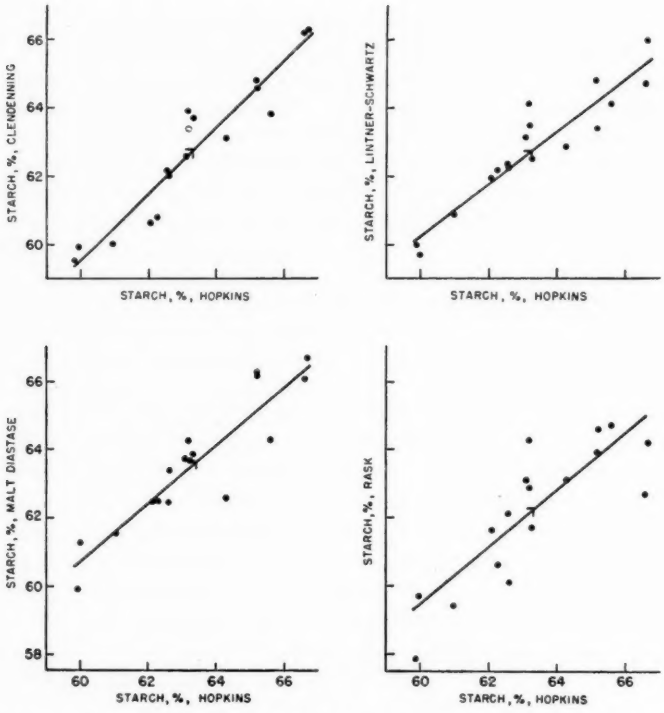

FIG. 1. *Scatter diagrams showing relations between starch content by the Hopkins method and starch content by other methods.*

TABLE I

CORRELATION COEFFICIENTS FOR EACH PAIR OF METHODS (n = 15)

Methods	Clendenning	Lintner–Schwarcz	Malt diastase	Rask
Hopkins	.95	.94	.92	.84
Clendenning		.93	.94	.82
Lintner–Schwarcz			.91	.88
Malt diastase				.81

content, the accuracy of the Rask method is adversely affected by some varying constituent (or constituents) other than starch.

The analysis of variance showed that the mean square due to the interaction between samples and methods was significantly greater than the mean square due to differences between duplicates (F = 2.9; required for 1% point, 1.8). This means that the methods do not place all samples in the same rank order, and that the discrepancies cannot be explained solely on

the grounds of experimental errors. In other words, real differences in the behaviour of some of the samples with different methods occur, and these must serve to reduce the correlation coefficients. Moreover, as such differential behaviour exists, it follows that the relative results given by any pair of methods cannot be determined by the study of two or three samples only, and that different investigators working with two or three samples each are likely to obtain different comparisons between a given pair of methods.

Correlations with Protein Content

It is interesting to consider whether any evidence can be advanced to show that any of the methods tends to overestimate low starch contents and underestimate high starch contents, or *vice versa*. It is difficult to determine this without knowing the true starch contents of the samples. Even if the Hopkins' values are accepted as true, the regressions for other methods on the Hopkins' method cannot be interpreted readily to give the required information. The regression coefficient falls as the correlation decreases, so that a direct comparison of regression coefficients is misleading unless the correlation coefficients are closely similar. This is not the case for the relations between the Hopkins' method and the other methods.

An attempt was made to overcome this difficulty by studying the relations between starch content and protein content for each method. The five scatter diagrams are shown in Fig. 2, together with a sixth diagram in which all the regression lines are collected. The correlation and regression coefficients are given in Table II. Here again the correlation coefficients differ

TABLE II

RELATIONS BETWEEN STARCH AND PROTEIN CONTENTS

Method	Correlation coefficient	Regression coefficient (starch on protein)
Hopkins	− .94	−0.90
Clendenning	− .94	−0.94
Lintner–Schwarcz	− .95	−0.75
Malt diastase	− .89	−0.80
Rask	− .87	−0.83

as between the polarimetric methods and the other two. However, among the polarimetric methods themselves the correlations are of the same order, and there is some difference among regression coefficients. Those for the Hopkins and Clendenning methods are not widely different, but that for the Lintner–Schwarcz method is much lower. Accordingly it may be suggested that if the Hopkins and Clendenning methods give results that are true over the whole range of starch values (except that the Hopkins values are always slightly higher than the Clendenning), then the Lintner–Schwarcz method tends to overestimate low starch content (high protein samples) and to underestimate high starch content. The two chemical methods may well do this also.

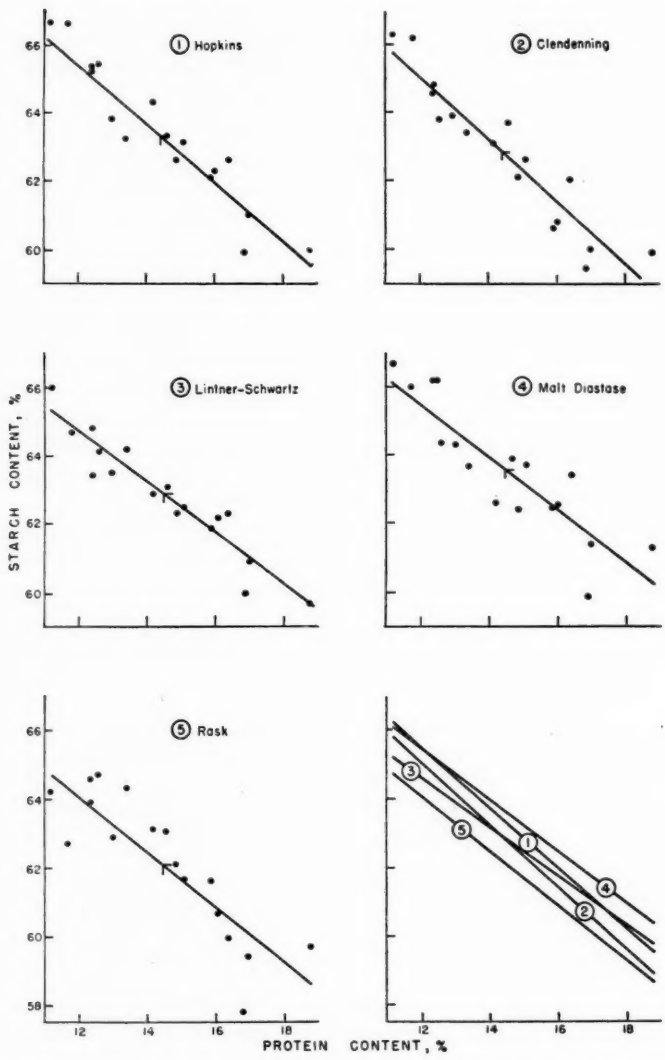

FIG. 2. *Scatter diagrams showing the relation between protein content and starch content as determined by each of five methods.*

It must be pointed out, however, that it was not possible to show that statistically significant differences occur between regression coefficients, though there was some evidence that this could be done with a larger number of samples.

Simplicity of Methods

The polarimetric methods are far simpler to handle than the chemical methods. Among the former, the Clendenning method is given first place. No preliminary washing is required, there is never any difficulty with frothing and burning, filtering presents no problem, and one operator can make 24 to 30 tests per day. In the Hopkins method, the washing is tedious and time consuming, and the digestion must be carefully watched, so that no more than 16 tests can be made in one day. The Lintner–Schwarcz method, with preliminary washing also required to obtain satisfactory filtration of wheat digestions, is in the same class as the Hopkins method. None of these three methods appears particularly difficult to learn.

Both the chemical methods are long and complicated. The malt–diastase method has too many steps and requires too much apparatus, and six determinations is a good day's work. Moreover, the amount of the sample represented in the final aliquot is small and errors are thus multiplied. The Rask method is also time consuming. It requires great attention to details of technique and is probably the most difficult method to master. However, in skilled hands it can be quite precise, and it has the advantage that it can be used in laboratories that have no polarimeter. But there is some doubt in our minds that the method can be depended upon to give accurate results even in the hands of a skilled operator.

General Conclusion

In this laboratory it is the intention to use the Clendenning method chiefly because of its greater speed and ease of operation. The method is not quite as precise as the Hopkins method and may be a little less accurate. But the relation between the two methods appears to be relatively constant over the starch range in which the laboratory is interested. Either method should therefore serve quite well for comparing samples.

Acknowledgments

The writers are indebted to Dr. J. A. Anderson for helpful advice and to Mr. W. O. S. Meredith for assistance with the statistics.

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EQUILIBRIUM DIAGRAMS FOR BINARY MIXTURES OF ANILINE, ETHYLANILINE, AND DIETHYLANILINE[1]

B. C. GREEN[2] AND J. W. T. SPINKS[3]

Abstract

Liquid–vapour composition curves have been determined for the systems aniline–monoethylaniline, monoethylaniline–diethylaniline, and aniline–diethylaniline. The experimental curve for aniline–monoethylaniline agrees quite well with the curve calculated using Raoult's law. However, the experimental curves for monoethylaniline–diethylaniline and aniline–diethylaniline show wide deviations from the calculated. From the shape of the experimental curves it appears that good separations of aniline, ethylaniline, and diethylaniline from one another may be effected by distillation with a reasonably good column. Improved separation could be obtained by distillation at reduced pressure.

Introduction

In many distillation problems it is necessary to know the composition of the vapour in equilibrium with a liquid at a given pressure. The relation between the composition of the liquid and the composition of the vapour is often expressed in a liquid–vapour composition diagram. Such a diagram is commonly called an equilibrium diagram and, in the following, equilibrium diagrams for several two component systems will be considered.

The experimental problem is to secure and analyse a sample of the vapour in equilibrium with a liquid mixture of known composition. The methods used for this purpose include:

(*a*) Compression of a gaseous mixture of known composition to its dew point,

(*b*) Distillation of a small portion of a liquid mixture of known composition and the analysis of the distillate,

(*c*) Direct analysis of the liquid and its equilibrium vapour,

(*d*) The saturation of an indifferent gas with the vapour of a binary mixture,

(*e*) Passing a mixed vapour of constant composition through a liquid mixture containing the same constituents,

(*f*) Distillation of a liquid mixture and the continuous recycling of the distillate back to the still.

The last method, which appears to have originated with Carveth (1), has been used in the present work. Liquid is boiled in a still (Fig. 1) and the condensed vapour collects in a trap. The overflow from the trap leads back to the still; this results in a recycling process. With continued boiling, equilibrium is reached and the temperature becomes steady. Under these

[1] *Manuscript received June 22, 1945.*
Contribution from the Department of Chemistry, University of Saskatchewan, Saskatoon, Sask.

[2] *Graduate student.*
[3] *Professor of Physical Chemistry.*

conditions, the distillate in the trap has the same composition as the vapour leaving the liquid. Distillation is stopped and the liquids in the trap and the still are analysed. The present form of the apparatus is due to Othmer (2).

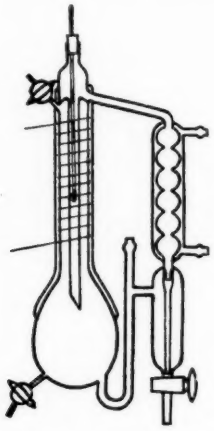

FIG. 1. *Diagram of apparatus.*

By lagging and electrically heating the parts of the apparatus carrying vapour to the condenser, rectification of the vapour by condensed liquid can be avoided (see also (4), (5), (6)).

Experimental

Apparatus

The apparatus used in the experiments was built around a 250 cc. Pyrex distilling flask, and is sufficiently explained by Fig. 1. The pressure during each run was kept constant with a manostat. The usual pressure was 760 mm. but a few runs were made at 331 mm.

The still was charged with about 100 cc. of liquid and heated with an electric heater. The heating current in the neck of the flask was adjusted until small variations in the rate of heating failed to produce variations in the thermometer readings. This method for compensating for heat losses was checked by experiments on pure aniline; found b.p. = 184.2° C., I.C.T. value = 184.4° C.

In the first type of apparatus used, temperatures were obtained from a thermometer immersed in a well that also served as a stopper for the flask. However, at 200° C. conductivity effects made accurate temperature measurements impossible with this type of apparatus, and the well was discarded. Thereafter, temperatures were obtained from a 360° C. partial immersion thermometer passing through a cork and in direct contact with the vapour.

The agreement between the observed boiling points of the pure liquids and the accepted values (Table I) indicates that the thermometry was satisfactory.

Distillation was continued for one hour, after which samples of liquid were withdrawn from both the trap and the still. The samples were kept in stoppered bottles until analysed.

Method of Analysis

Samples were analysed by the refractive index method using a Pulfrich refractometer (with temperature control) and a sodium vapour lamp. After determining the refractive index, n_D^{20}, the composition could be read off from a plot of refractive index vs. percentage composition. This plot had been previously constructed from data obtained for known mixtures made up by weight from purified materials.

Reagents

The benzene and carbon tetrachloride were of Merck Reagent quality. The samples of aniline, ethylaniline, and diethylaniline were dried over potassium hydroxide and distilled. Four fractions of each were collected. The refractive indices of the last three portions differed from the refractive index of the first but agreed among themselves. These last three fractions were mixed and again distilled, the first few cubic centimetres being discarded. The resulting distillate was used for the test runs and the calibration samples.

TABLE I

CONSTANTS OF REAGENTS

Material	B.p. (760mm.), °C.	d_4^{20}	n_D^{20}	Source of data
Aniline	184.4	1.022	1.5863	I.C.T.*
	184.2	1.022	1.5859	Experimental
Ethylaniline	204.7	0.963	1.5559	I.C.T.
	206.0	0.963	—	C.E.H.†
	205.0	0.960	1.5541	Experimental
Diethylaniline	216.3	0.934	1.5421	I.C.T.
	217.0	0.935	—	C.E.H.
	217.0	0.934	1.5415	Experimental

* *International Critical Tables.* † *Chemical Engineering Handbook.*

Results

Preliminary runs were made on benzene–acetic-acid and on benzene–carbon-tetrachloride mixtures. The results agreed very well with those given in the literature (2, 3), and consequently the apparatus was considered to be operating satisfactorily (details of these experiments have been recorded).*

* *Green, B.C. Thesis, University of Saskatchewan, 1942.*

Runs were then made on the three systems, aniline-ethylaniline, ethylaniline-diethylaniline and aniline-diethylaniline. Most of the experiments were done at 760 mm. pressure; a few, at 331 mm. pressure. The data are recorded in Tables II and III, and presented graphically in Figs. 2 and 3.

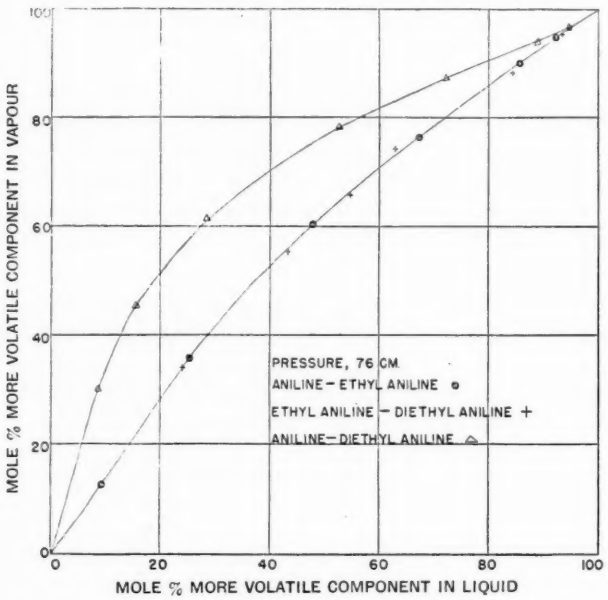

FIG. 2. *Equilibrium diagram for binary mixtures of aniline, ethylaniline, and diethylaniline.*

TABLE II.

VAPOUR–LIQUID COMPOSITIONS FOR ANILINE-ETHYLANILINE, ETHYLANILINE–DIETHYLANILINE AND ANILINE–DIETHYLANILINE

Pressure, cm.	Aniline–ethylaniline		Ethylaniline–diethylaniline		Aniline–diethylaniline	
	Mole % aniline		Mole % ethylaniline		Mole % aniline	
	Liquid	Vapour	Liquid	Vapour	Liquid	Vapour
76.0	9.3	12.5	24.1	34.0	8.7	30.1
76.0	25.1	35.8	43.3	55.3	15.5	45.4
76.0	47.7	60.4	54.8	65.7	28.6	61.4
76.0	67.4	76.4	63.0	74.2	52.7	78.1
76.0	85.6	90.1	84.4	88.3	72.4	87.4
76.0	92.4	95.0	93.4	95.5	89.0	93.9
76.0	—	—	—	—	94.7	96.8
33.1	45.5	59.8	51.2	65.6	52.7	77.4
33.1	68.8	79.1	88.6	95.1	75.8	89.0
33.1	84.3	89.8	—	—	—	—

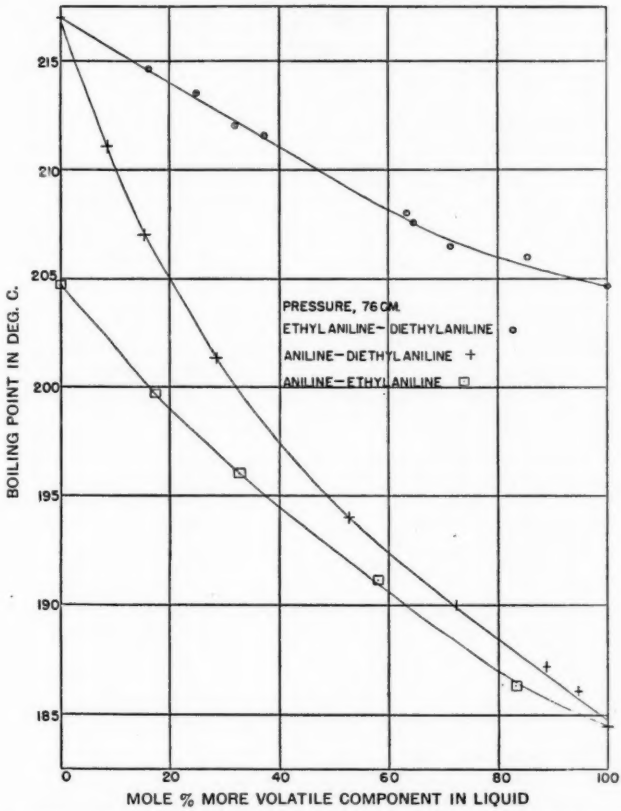

FIG. 3. *Boiling points of binary mixtures of aniline, ethylaniline, and diethylaniline.*

TABLE III

VARIATION OF BOILING POINT WITH COMPOSITION
Pressure, 76 cm.

Aniline–ethylaniline		Aniline–diethylaniline		Ethylaniline–diethylaniline	
Mole % aniline	B.p., ° C.	Mole % aniline	B.p., ° C.	Mole % ethylaniline	B.p., ° C.
100	184.4	100	184.4	100	204.7
83.3	186.3	94.7	186.0	85.3	206.0
58.0	191.1	89.0	187.2	71.4	206.5
32.8	196.0	72.4	190.0	64.3	207.6
17.5	199.7	52.7	194.0	63.4	208.0
0	204.7	28.6	201.3	37.2	211.5
—	—	15.5	207.0	31.9	212.0
—	—	8.7	211.0	25.0	213.5
—	—	0	217.0	16.0	214.6
—	—	—	—	0	217.0

Discussion

When accurate vapour pressure data are available, liquid–vapour composition diagrams may be calculated using Raoult's law. Using the vapour pressure data in the International Critical Tables, Vol. 3, p. 221, the calculated equilibrium curve for aniline-monoethylaniline agrees quite well with the experimental. However, the calculated curves for monoethylaniline-diethylaniline and aniline-diethylaniline show wide deviations from the experimental. The extent to which the deviations are real is not known since some of the vapour pressures used in the calculations are obtained by an extrapolation and may therefore be in error.

From the shape of the experimental curves it appears that good separations of aniline, ethylaniline, and diethylaniline from one another may be effected by distillation with a reasonably good column*. The results at low pressures indicate that, in distillation, improved separation could be obtained at diminished pressure. Diminished pressure would have the further advantage of lower temperature and less decomposition. Coloured products are formed by distillation at atmospheric pressure, even when air is excluded, and thus a lower temperature (with decreased decomposition) is desirable.

Acknowledgments

Thanks are here expressed to Shawinigan Chemicals Limited, for a sample of monoethylaniline, and for helpful correspondence, and to Mr. W. Graham, for boiling point measurements on ethylaniline-diethylaniline and aniline-ethylaniline.

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* *See footnote, p. 271.*

DETERMINATION OF FLUORIDES IN WATER BY MEANS OF A PHOTOELECTRIC COLORIMETER[1]

OSMAN JAMES WALKER[2] AND GORDON CLEMENTS GAINER[3]

Abstract

A method has been developed for the determination of small amounts of fluorides by means of a photoelectric colorimeter. The method is based on the bleaching of the lake formed from a zirconyl salt and sodium alizarin sulphonate by the action of fluoride ion. The photoelectric colorimeter used is of the direct reading type based on that developed by Evelyn but differs in that the light passes vertically through a long absorption cell rather than horizontally through an absorption cell consisting of a large test-tube. By calibrating the galvanometer scale against solutions containing known amounts of fluoride, the number of parts per million of fluorides in water can be determined. A comparison of the results obtained in the photoelectric colorimeter with those from the corresponding visual method show that the two sets of data are in agreement with each other.

During recent years the photoelectric colorimeter has been adapted for the quantitative analysis of a considerable number of substances. Up to the present very little has been done to make use of this instrument in the determination of small amounts of fluorides. Wilcox (6) described a method in which he made use of the fading action of fluorine on the colour of ferric acetyl acetone, this being the modification of an earlier procedure developed by Armstrong (1). Shvedov (3) developed a photoelectric colorimeter that he used successfully in determining small amounts of fluorine. He described one method based on the bleaching effect of fluorine on a "peroxidized" solution of a titanium salt, and another in which he utilized the bleaching action of fluorides on the red lake formed by zirconyl nitrate and sodium alizarin sulphonate. Urech (4) made use of the action of fluorides on the coloured compound formed between ferric ion and ferron. Each of the investigators used different types of instruments some of which were quite complicated.

The fact that so few papers have been published on the determination of fluorides by electro-optical methods in comparison with those concerned with visual methods seems to indicate that there might be some inherent difficulties involved in adapting some of the well established procedures to the new technique.

In these laboratories a great deal of attention has been given to the determination of fluorine in the water supplies of Alberta. The method that has been used to the greatest extent (5) involves the fading effect of fluorides on the lake formed by zirconyl nitrate and sodium alizarin sulphonate. This is one of the most rapid and accurate colorimetric methods for the determina-

[1] *Manuscript received July 21, 1945.*
Contribution from the Department of Chemistry, University of Alberta, Edmonton, Alta.
[2] *Professor of Chemistry.*
[3] *Honours Student, 1942.*

tion of small amounts of fluorine and has possibilities in so far as it might be adapted to a photocolorimetric method. There is, however, a possible disadvantage in that the colour of the lake is rose red, which in the presence of fluorides goes over to the yellow colour of the sodium alizarin sulphonate probably owing to the formation of the stable ZrF_6^{--} ion. With intermediate amounts of fluoride, colours of the solutions are from red to yellow. This method can be used only for amounts of fluoride ion from 0.01 to 0.15 mgm. in 100 cc. of water. This corresponds to 0.1 to 1.5 parts of fluorine per million parts (p.p.m.) of water.

In order to determine whether the reaction might be of value from the photoelectric standpoint, the absorption spectrum of the red lake was measured in a Hilger–Nutting visual spectrophotometer. The solution was made up as follows: 4.0 cc. of standard stock solution containing 0.87 gm. of $ZrO(NO_3)_2.2H_2O$ and 0.17 gm. of sodium alizarin sulphonate per cc. was added to 100 cc. of distilled water along with 2.0 cc. of 3 *N* hydrochloric acid and 2.0 cc. of 3 *N* sulphuric acid; this was boiled, allowed to stand overnight and made up to 100 cc. In Fig. 1, the extinction coefficient is plotted against wave-length in millimicrons, Curve *A* being for the red lake. It will be seen that there is strong absorption in the vicinity of 520 mμ where absorption is at a maximum. In order to ascertain whether there might be any interference from the yellow sodium alizarin sulphonate, the examination of a solution of this substance of the same concentration as used in the previous experiment was carried out. Curve *B* in Fig. 1 shows the results. It will be seen that there is some overlapping in the two curves but the maximum absorption of the latter is somewhere in the ultra-violet portion of the spectrum. It was thought that a satisfactory photoelectric method might be developed.

Construction of a Photoelectric Colorimeter

In order to carry out the measurements a photoelectric colorimeter was constructed in accordance with the directions worked out by Evelyn (2). The diagram in Fig. 2 is a schematic representation of the instrument. The case for holding the colorimeter is not shown.

Description of Apparatus

The light source, *L*, consisted of a flash light bulb energized by a six-volt storage battery, the intensity of the beam being controlled by fixed and variable resistances. The colour filter, *F*, was a Wratten gelatine filter, 61 *N*, manufactured by the Eastman Kodak Company, which had a maximum transmission at 525 mμ not far from 520 mμ, which was the wave-length for maximum absorption by the red lake of zirconium sodium alizarin sulphonate.

In nearly all photoelectric colorimeters the light beam passes horizontally through a layer of the coloured liquid several centimetres in thickness. This is true of the Evelyn instrument (2), which makes use of a test-tube $\frac{7}{8}$ in. in diameter. This was found to be lacking in sensitivity for the determination of fluorides by the method used. For this reason the construction of the

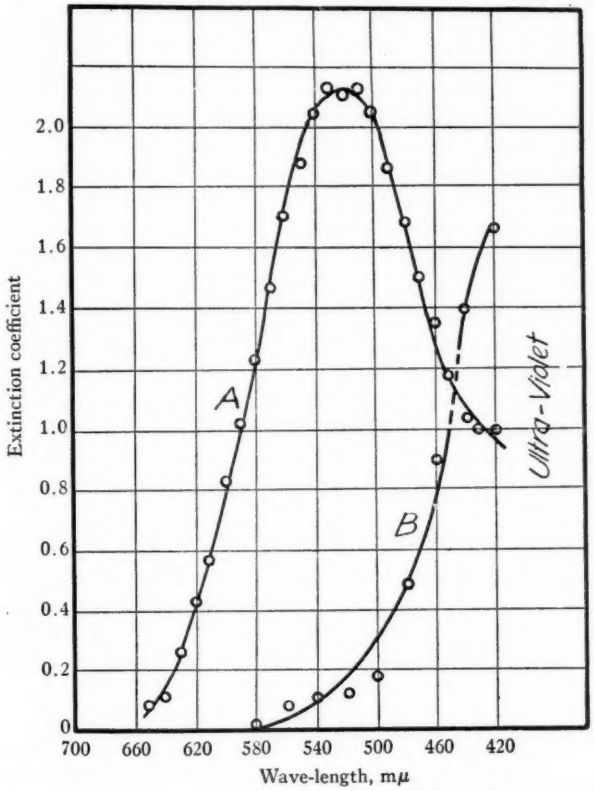

FIG. 1. *Absorption spectra of sodium alizarin sulphonate and of the* **red lake.**

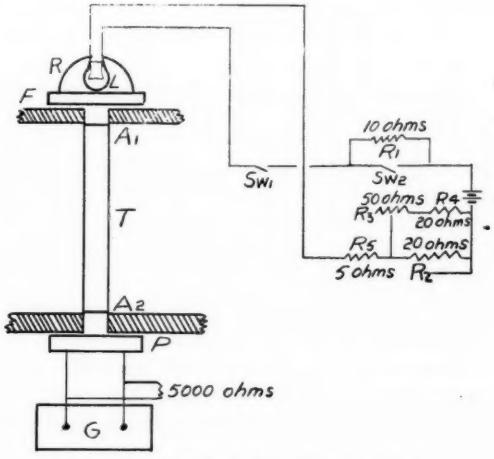

FIG. 2. *Assembly for photoelectric colorimeter.*

colorimeter was so modified as to permit the use of a long absorption cell, viewed longitudinally by the photocell instead of across the diameter. The absorption cell, *T*, consisted of a Nessler tube $\frac{3}{4}$ in. in diameter and 21.5 cm. long. The upper surface had on it a ground edge. The tube is filled with the solution and an 18 mm. microscope cover slip is placed on top. It has been found that the surface tension of the solution pulls on the cover slip and holds it tightly in place. Care should be taken to see that no salt smears or finger prints are on the cover slip surface or on the bottom or walls of the tube. Several absorption tubes should be available. In order for any two tubes to give the same reading or be interchangeable, they must be of the same length, and have the same diameter and the same condition of outside surface.

The photocell, *P*, was of General Electric Company manufacture, catalogue No. 88–565. The spectral response of this light-sensitive cell was from 320 mμ to 640 mμ. The galvanometer, *G*, with an external damping resistance of 5000 ohms, was constructed in the laboratory and used with a 2.5 m. light path. Its sensitivity was 2.5×10^{-6} amp. per 100 scale divisions. Duplicate readings agreed to about one-half a scale division.

Calibration and Operation of the Photoelectric Colorimeter

The photoelectric colorimeter was calibrated by using concentrations of fluoride from 0.2 to 1.5 p.p.m. treated by the method of Walker and Finlay (5). Six runs on different solutions were made at each concentration. The galvanometer readings were averaged and are presented in Table I. In

TABLE I

AVERAGE GALVANOMETER READINGS WITH SOLUTION OF DEFINITE CONCENTRATIONS OF F^-

Conc. of F^- in p.p.m.	Average galvanometer readings	Conc. of F^- in p.p.m.	Average galvanometer readings
0.2	38.9	1.0	49.5
0.4	40.6	1.2	53.8
0.6	42.3	1.4	59.7
0.8	46.4	1.5	60.1

Fig. 3, the average galvanometer readings are plotted against fluoride content in parts per million and the best fit curve was constructed. This curve is not a straight line as would be the case if the Lambert–Beer law held for this system. The failure of this law to apply is undoubtedly due to the presence in the system not only of the red lake of zirconyl sodium alizarin sulphonate but also of simple sodium alizarin sulphonate, both of which have their own characteristic absorption bands (see Fig. 1).

It will be noted that values for solutions of fluoride ion of greater concentrations than 1.5 p.p.m. have not been determined. This is because of the fact that the red lake formed from zirconyl nitrate and sodium alizarin sulphonate

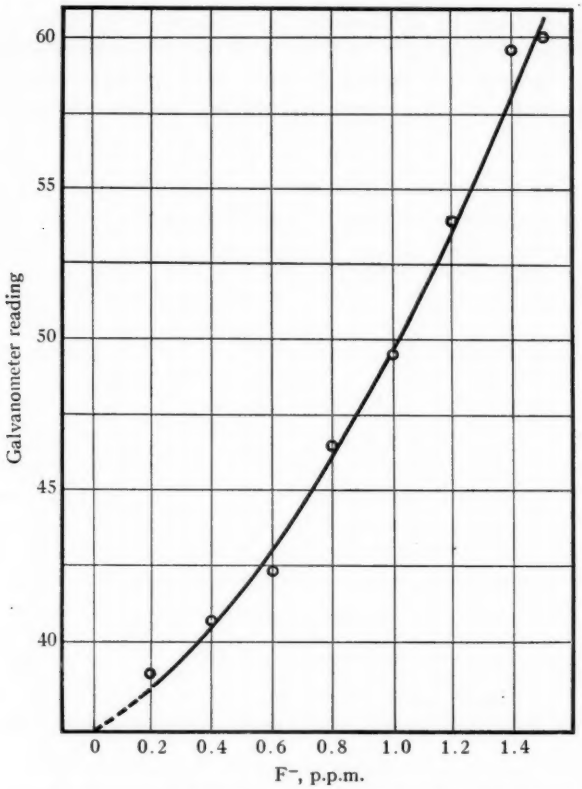

FIG. 3. *Conversion of galvanometer readings into parts per million of* F^-.

is bleached to too great an extent when 1.5 p.p.m. or more of F^- is present. If the concentration of the fluoride in the water supply is greater than this, it is customary to start with a smaller sample and dilute to 100 cc. with distilled water. This is taken into account in the subsequent calculations.

Photoelectric Determination of Fluorides in Water

In order to check the behaviour of the photoelectric colorimeter a number of samples of Alberta waters were analysed. The method of analysis used was the one described by Walker and Finlay (5). In all cases the sulphate content of the water was also determined and corrections where made according to the procedure presented in the above paper. Determinations were carried out by both the visual method and by the photoelectric method, in which concentrations of fluoride ion were calculated from the galvanometer readings using Fig. 3 and then correcting for sulphate content where necessary. With waters high in fluorides the appropriate size of sample was used and diluted to 100 cc. with distilled water as explained earlier.

Values obtained are shown in Table II. In Columns 1 and 4 are given the laboratory numbers of the sample, in Columns 2 and 5 the values obtained by the visual method, and in Columns 3 and 6 the values obtained by the photoelectric method. It will be seen that the two methods check with each other fairly well.

TABLE II

FLUORINE CONTENT OF NATURAL WATERS IN PARTS PER MILLION

Laboratory numbers	F^-, visual	F^-, photoelectric	Laboratory numbers	F^-, visual	F^-, photoelectric
1093	1.4	1.3	1108	1.1	1.15
1094	1.1	1.1	1109	0.5	0.5
1095	0.4–0.5	0.4	1111	0.6	0.7
1096	1.5	1.5	1112	0.2	0.2
1097	1.2	1.35	1113	0.5	0.5
1098	1.5	1.5	1114	0.5	0.4
1099	1.1	1.0	1115	1.3	1.35
1100	1.2	1.35	1117	0.9	0.9
1102	1.3	1.4	1118	1.1–1.2	1.3
1103	1.0	1.0	1119	1.1	1.05
1104	0.9	0.9	1123	1.1	1.05
1105	0.8	0.8	1124	0.5	0.5
1107	1.5–1.6	1.6	1125	1.5	1.5

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PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

VI. DEHYDRATION BY SULPHURIC ACID[1]

By A. C. Neish[2], V. C. Haskell[3], and F. J. Macdonald[2]

Abstract

Dehydration of 2,3-butanediol in the liquid phase with 3 to 20% of sulphuric acid gave water, butanone-2, and the cyclic methyl ethyl ketal of 2,3-butanediol as the chief products, the total yield of liquid products being about 96%. The reaction can be run as a continuous process, and by using conditions that favour removal of the cyclic ketal from the reaction mixture it may make up 75% of the weight of the total liquid products. The rate of dehydration of 2,3-butanediol varied as the square of the 2,3-butanediol concentration and increased logarithmically with increase in the concentration of sulphuric acid.

Introduction

The dehydration of 2,3-butanediol has received considerable study, since butadiene is to be expected as a product. However, all attempts to convert it to butadiene, in high yields, by direct dehydration have been unsuccessful since butanone-2 is obtained as the main product. Backer (1) also obtained the cyclic methyl ethyl ketal of 2,3-butanediol by passing the diol vapour over an alumina dehydration catalyst.

The writers have investigated the dehydration of 2,3-butanediol in the liquid phase, using sulphuric acid as the catalyst, in order to see what yields of the above products could be obtained under varying conditions. This reaction was selected for study because it would be expected to yield fairly pure products from relatively crude 2,3-butanediol produced by fermentation.

Experimental

The dehydration of *l*-2,3-butanediol by sulphuric acid was studied in two different ways. (*a*) Mixtures of sulphuric acid and 2,3-butanediol were heated in a distillation apparatus that permitted the products, but not the reactants, to distil out; the volume of the reaction mixture was kept constant by feeding in 2,3-butanediol to replace that used up. (*b*) Mixtures of sulphuric acid and 2,3-butanediol were heated in sealed tubes containing a relatively small vapour space; under these conditions the products remain chiefly in the liquid phase.

(1) *Dehydration of 2,3-Butanediol with Continuous Removal of Products*

The apparatus used consisted of a 300 ml. Kjeldahl flask sealed to a condenser; the neck of the flask was packed with glass helices and insulated with

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asbestos. Two platinum electrodes were sealed in the wall of the flask, as well as a short tube to admit fresh 2,3-butanediol. A thermometer was arranged to dip into the reacting mixture. At the start of an experiment the flask was charged with a mixture of sulphuric acid and 2,3-butanediol and connected to a 2,3-butanediol reservoir through a solenoid valve. It was then heated in a constant temperature bath regulated within $\pm 1°$ C. The products distilled out as they formed and after a steady state was reached they collected at a constant rate in the receiver; no appreciable amounts of 2,3-butanediol or sulphuric acid came over. The reaction mixture was kept at constant volume since when the liquid level fell slightly the electrodes were exposed, thus breaking a relay circuit; this caused the solenoid valve to open and allow fresh 2,3-butanediol to flow in to replace that used up. As a result the concentration of sulphuric acid was constant during any given experiment. The products were fractionated through a Stedman column (18 in. long). The main fractions obtained were the butanone-2 water azeotrope (b.p. 73° to 74° C.) and a higher boiling liquid (b.p. 133° to 134° C.). The latter fraction was immiscible with water and had a camphor-like odour. It contained an impurity that decolorized cold, neutral permanganate solution but this was removed, by a second fractionation, as a lower boiling fraction.

The purified fraction was analysed: C, 66.65, 66.66; H, 11.25, 11.14%. These values agree with the theoretical values for the cyclic ketal formed between butanone-2 and 2,3-butanediol (C, 66.61; H, 11.19%). It was shown to be this by hydrolysis to, and synthesis from, 2,3-butanediol and butanone-2. The butanone-2 was identified by the melting point of its 2,4-dinitrophenylhydrazone, a mixed melting point being determined with an authentic sample.

(2) *Hydrolysis of the Cyclic Ketal*

The liquid fraction (300 gm.), b.p. 133° to 134° C., obtained by dehydration of *l*-2,3-butanediol with sulphuric acid was refluxed with aqueous 1.3% hydrochloric acid (100 gm.) for 45 min. The mixture was then neutralized with normal sodium hydroxide. It formed two layers, which were separated and each fractionated through a Stedman column. The hydrolysis was about 60% complete, the products being butanone-2 and 2,3-butanediol in equimolecular quantities. The 2,3-butanediol recovered was slightly isomerized, having $[\alpha]_D^{25} = -10.4°$, and a refractive index of 1.4320 at 25° C.

(3) *Synthesis of the Cyclic Ketal*

Pure *l*-2,3-butanediol (90 gm.) and anhydrous butanone-2 (80 gm.) were mixed in a container cooled in an ice-bath, and dry hydrochloric acid was bubbled in slowly until the reaction mixture separated into two layers. The top layer was washed with water, dried over anhydrous sodium sulphate, and fractionated through a Stedman column. About 56 gm. of material boiling at 133° to 134° C. was obtained. This is the *levo*-isomer of the cyclic ketal formed between 2,3-butanediol and butanone-2. Calc.: C, 66.61; H, 11.19%. Found: C, 66.61; H, 11.25%. Its physical properties were compared

(Table I) with those of the compound obtained on treating *l*-2,3-butanediol with sulphuric acid. The two compounds are identical except the latter seems to be partly isomerized. It is possible that both show some isomerization, since acid was used in each synthesis, but the *levo*-isomer is predominant.

(4) *Dehydration of 2,3-Butanediol by Sulphuric Acid*

Mixtures of 2,3-butanediol and sulphuric acid (3 ml.) were sealed in tubes of about 5 ml. capacity made from 10 mm. diam. Pyrex tubing. These tubes were heated in a constant temperature wax bath regulated within $\pm 0.5°$ C., removed at definite time intervals, cooled to stop the reaction, and the contents analysed for 2,3-butanediol and the cyclic ketal by the methods described below. The kinetics of the dehydration of 2,3-butanediol by sulphuric acid was studied in this manner. Since it was found that only a small amount of the butanediol (about 10%) is converted to the cyclic ketal quickly, and that this stays relatively constant, the reaction being measured is the dehydration of 2,3-butanediol to butanone-2.

(5) *Estimation of 2,3-Butanediol and the Cyclic Ketal*

Both of these compounds reduce periodic acid quantitatively. Advantage is taken of their different solubilities to determine them in the presence of each other. The cyclic ketal is only slightly soluble in water while 2,3-butanediol is hygroscopic. To determine both in the same mixture it is dissolved in about 200 parts of water and the total periodate reducing value of this solution is determined before and after a single extraction with an equal volume of isopropyl ether at room temperature. By reference to a graph constructed from known mixtures of these compounds the percentage of the total reducing value due to 2,3-butanediol is obtained, and hence the total amount of each compound present can be readily calculated. The graph was made by plotting percentage of total reducing value not extracted by isopropyl ether against the percentage of 2,3-butanediol in the mixture.

(6) *Determination of Equilibrium Constants*

A mixture of 2,3-butanediol (1 mole), butanone-2 (1 mole), and water (0.5 mole) was prepared, 2.7 ml. aliquots were measured into small test-tubes, 0.3 ml. of sulphuric acid was added, and the tubes were placed in a constant temperature bath. They were shaken at frequent intervals, and a tube was removed from time to time for analysis. When the concentration of ketal had reached a steady value the concentrations of the reactants and products were estimated and the value of the equilibrium constant was calculated from the data, following the usual conventions.

Results

No butadiene was formed in these experiments but it was possible to obtain either butanone-2 or the cyclic methyl ethyl ketal of 2,3-butanediol as the chief product by varying the experimental conditions.

TABLE I

COMPARISON OF SAMPLES OF THE CYCLIC METHYL ETHYL KETAL OF *l*-2,3-BUTANEDIOL MADE BY DIFFERENT METHODS

Property	Product obtained by distillation of a mixture of sulphuric acid and 2,3-butanediol	Product obtained by treating a mixture of *l*-2,3-butanediol and butanone-2 with dry hydrogen chloride at room temperature
Refractive index at 25° C.	1.4054	1.4051
Density at 25° C.	0.8708	0.8707
Optical rotation: $[\alpha]_D^{25}$	−15.00°	−18.75°
Boiling point	133.1° C. (749 mm.)	133.0° C. (755 mm.)

The cyclic ketal obtained was chiefly the *levo*-isomer (b. p. 133° to 134°C.) since *levo*-2,3-butanediol was used. It was identified by hydrolysis, synthesis, and analysis (see Experimental). The product synthesized from *levo*-2,3-butanediol and butanone-2 had the same physical properties as that obtained by heating *levo*-2,3-butanediol with sulphuric acid except for a higher optical rotation (see Table I). The hot acid probably causes some isomerization. The cyclic ketal obtained by Backer (1) had a boiling point of 141° to 143° C. and was chiefly the *meso*-isomer since he used *meso-dextro* 2,3-butanediol.

Study of the experimental conditions showed that the cyclic ketal is obtained in appreciable amounts only when its rapid removal from the reaction mixture is favoured. Thus it was found (Table II) that raising the temperature increased the yield of ketal at atmospheric pressure (compare Expts. 2, 3, 4, 5, and also 6, 7, 8). However, lowering the pressure gave even higher yields at lower temperatures (Expts. 9, 10) and in this way it was possible to obtain 95% of the theoretically possible yield (Expt. 10). Addition of water to the feed slightly increased the yield of ketal (compare Expts. 3, 11, 12) probably because it favoured its removal by a steam distillation. It is also possible to raise the actual yield of ketal per mole of 2,3-butanediol by adding butanone-2 to the feed (Expts. 13, 14, 15) and in this case also the yield of ketal is increased by lowering the pressure. The space-time yield data in Table II show how these various conditions influence the rate of the reaction.

It was possible to study the kinetics of the dehydration of 2,3-butanediol to butanone-2 without interference from the reaction that results in ketal formation, since in experiments where escape of products from the liquid phase is prevented a small amount of the cyclic ketal forms quickly and then stays at an approximately constant level during the course of the reaction. Mixtures of 2,3-butanediol and sulphuric acid were heated in sealed tubes and the rate of change of 2,3-butanediol concentration was followed. Twenty-one experiments of this type were made at various concentrations of sulphuric acid and at

TABLE II

DEHYDRATION OF 2,3-BUTANEDIOL BY DISTILLATION (SULPHURIC ACID CATALYST)

Expt. No.	Composition of feed*	% by wt. of sulphuric acid	Temp. of reacting mixture, °C.	Pressure, mm. of Hg	Space–time yield of products†	Moles of cyclic ketal per mole of diol	Yield of cyclic ketal as % of theoretical‡
1	2,3-Butanediol (99%) + water (1%)	5.5	126	Atmospheric	10.4	0.078	15.6
2	2,3-Butanediol (99%) + water (1%)	10.0	120	Atmospheric	7.5	0.053	11.6
3	2,3-Butanediol (99%) + water (1%)	10.0	128	Atmospheric	32.0	0.200	40.0
4	2,3-Butanediol (99%) + water (1%)	10.0	133	Atmospheric	53.6	0.240	48.0
5	2,3-Butanediol (99%) + water (1%)	10.0	138	Atmospheric	194.0	0.320	64.0
6	2,3-Butanediol (99%) + water (1%)	20.0	120	Atmospheric	17.4	0.067	13.4
7	2,3-Butanediol (99%) + water (1%)	20.0	125	Atmospheric	66.0	0.249	49.8
8	2,3-Butanediol (99%) + water (1%)	20.0	131	Atmospheric	160.0	0.290	58.0
9	2,3-Butanediol (99%) + water (1%)	10.0	120	150	109.0	0.376	75.2
10	2,3-Butanediol (99%) + water (1%)	10.0	113	123	48.3	0.477	95.4
11	2,3-Butanediol (80%) + water (20%)	10.0	128	Atmospheric	38.1	0.243	48.6
12	2,3-Butanediol (50%) + water (50%)	10.0	128	Atmospheric	52.5	0.258	51.6
13	2,3-Butanediol (80%) + butanone-2 (20%)	10.0	138	Atmospheric	265.0	0.330	51.7
14	2,3-Butanediol (50%) + butanone-2 (50%)	10.0	138	Atmospheric	360.0	0.511	51.1
15	2,3-Butanediol (50%) + butanone-2 (50%)	10.0	85	125	292.0	0.631	63.1

* *The butanone-2 added was the azeotropic mixture containing 11% of water.*

† *Grams of distillate obtained per 100 ml. of reacting mixture per hour (includes water and butanone-2 when present in feed).*

‡ *Calculated on the assumption that butanone-2, when present in feed, could combine with an equimolecular amount of 2,3-butanediol to give an equimolecular amount of the cyclic ketal.*

several temperatures. It was found, in all cases, that the reciprocal of the concentration of 2,3-butanediol plotted against time gave a linear relation. Hence the rate of the reaction appears to be proportional to the square of the concentration of 2,3-butanediol. The results of some typical experiments are shown in Fig. 1; the slope of the line gives the rate constant. The results

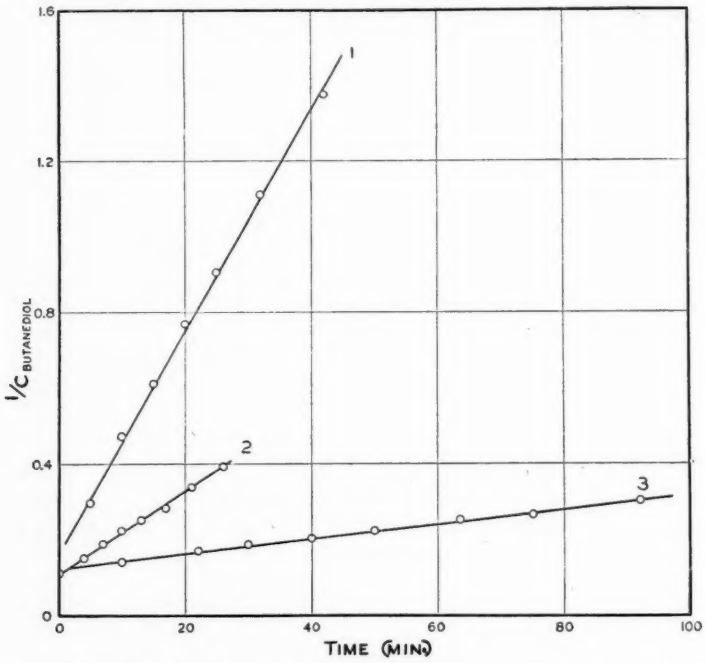


FIG. 1. *Variation of the rate of dehydration with the concentration of 2,3-butanediol. Mixtures of 2,3-butanediol and sulphuric acid were heated in sealed tubes and the change in 2,3-butanediol concentration with time was followed.*

1. Temperature 185° C.; 0.521 moles per litre of sulphuric acid.
2. Temperature 161° C.; 1.067 moles per litre of sulphuric acid.
3. Temperature 145° C.; 1.070 moles per litre of sulphuric acid.

The reaction being measured is the conversion of 2,3-butanediol. The rate constant is calculated from the slope of the line. The reciprocal of the butanediol concentration is plotted as litres per mole on the ordinate.

of all these experiments are summarized in Fig. 2. The rate of the reaction is quite sensitive to the concentration of sulphuric acid, and over most of the range studied it increased logarithmically with increasing sulphuric acid concentration. The rate varies rapidly with temperature and obeys the Arrhenius relation over most of the range (Fig. 3) but deviates slightly at the lower temperatures. Increasing the temperature 10° C. approximately trebles the rate of the reaction.

The formation of the cyclic ketal from butanone-2 and 2,3-butanediol in the presence of sulphuric acid was studied at low temperatures. This is a reversible reaction. The equilibrium constant was found to be 4.38 at 0.8° C. and 1.76 at 26° C. (17.9% of sulphuric acid as catalyst.) Thus the equilibrium shifts in favour of ketal formation as the temperature is lowered.

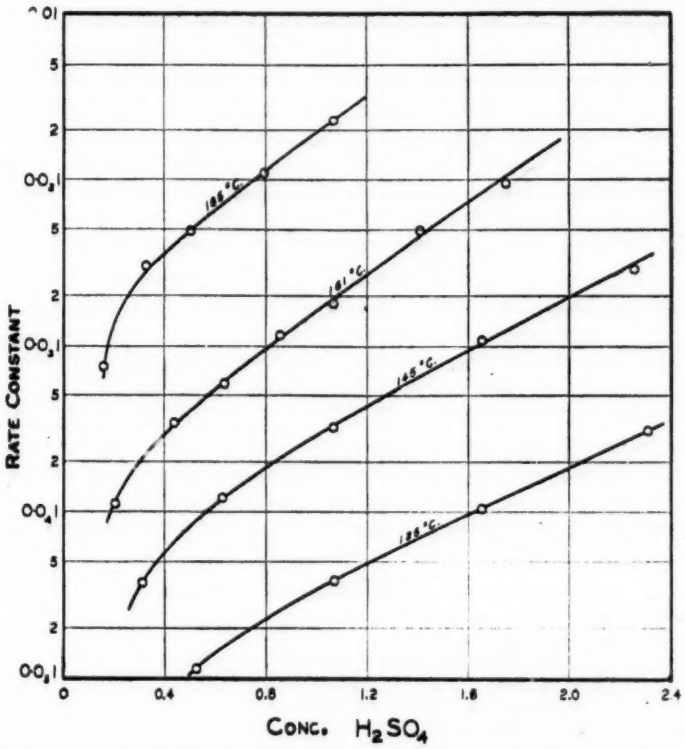


FIG. 2. *Variation of the rate of dehydration of 2,3-butanediol with the concentration of sulphuric acid.*

The rate of conversion of 2,3-butanediol to butanone-2 was measured. The rate constant, calculated as in Fig. 1, is expressed as litres per mole per second on the ordinate. The concentration of sulphuric acid is plotted as moles per litre on the abscissa.

Discussion

The results obtained can be explained by assuming the following reactions occur when 2,3-butanediol is heated with sulphuric acid:

(*a*)

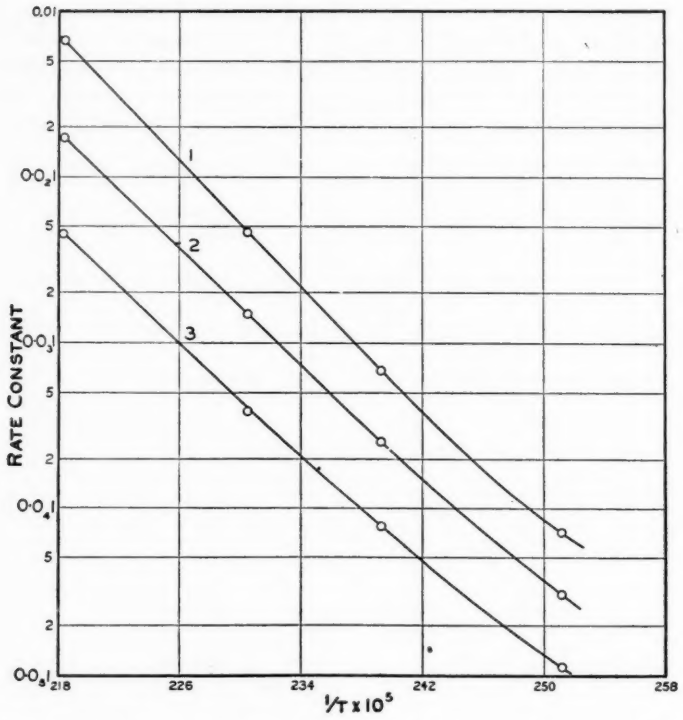



FIG. 3. *Variation of the rate of dehydration of 2,3-butanediol to butanone-2 with temperature.*

Plotted from data in Fig. 2.

1. 1.44 moles of sulphuric acid per litre.
2. 0.96 moles of sulphuric acid per litre.
3. 0.48 moles of sulphuric acid per litre.

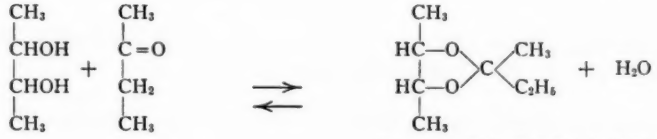
The rate constant is expressed as litres per mole per second.

(*b*) $CH_3CHOH\cdot CHOH\cdot CH_3 + CH_3CHOSO_2OH\cdot CHOH\cdot CH_3 \rightleftharpoons CH_3CHOSO_2OCH\cdot CHOH\cdot CH_3 + H_2O$

(*c*) $CH_3CHOSO_2OCH\cdot CHOH\cdot CH_3 \rightarrow CH_3CHOSO_2OH\cdot CHOH\cdot CH_3 + CH_3C{=}O\cdot CH_2\cdot CH_3$ Butanone-2

(*d*) $H_2SO_4 + n\,H_2O \rightleftharpoons H_2SO_4 \cdot n\,H_2O$

(*e*)



Cyclic methyl ethyl ketal of 2,3-butanediol

Application of the law of mass action to Reactions (*a*), (*b*), and (*c*) leads to the conclusion that the rate of butanone-2 formation should vary directly with the square of the 2,3-butanediol concentration and inversely with the concentration of water. Since no retarding effect by water was observed it is necessary to assume it is removed by Reaction (*d*), where, in the most extreme case studied, $n = 10$. Sulphuric acid would be expected to favour butanone-2 formation by its mass action in Reaction (*a*) and by removing water as in Reaction (*d*), so it is not surprising that the rate of the reaction is very sensitive to changes in the concentration of sulphuric acid.

The data in Table II can be explained by considering the equilibrium (*e*). Raising the temperature at atmospheric pressure increases the yield of the cyclic ketal since it favours removal of the products from the reaction mixture, so a larger proportion of the butanone-2 formed in Reaction (*c*) tends to form the ketal. However, raising the temperature causes the equilibrium (*e*) to shift to the left so when the removal of the products is accomplished by lowering the pressure, rather than by raising the temperature, the best yields of ketal are obtained. The slight increase in ketal formation obtained on adding water to the feed is probably caused by its removal by steam distillation.

Reference

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DISSIMILATION OF GLUCOSE BY *BACILLUS SUBTILIS* (FORD'S STRAIN)[1]

By A. C. Neish[2], A. C. Blackwood[2], and G. A. Ledingham[2]

Abstract

Ford's strain of *Bacillus subtilis* (N.C.T.C. 2586) dissimilated glucose mainly to 2,3-butanediol and glycerol under anaerobic conditions at pH 6.2 to 6.8. For each 100 moles of glucose fermented, 57 moles of 2,3-butanediol, 40 moles of glycerol, 20 moles of lactic acid, 13 moles of ethanol, and 5 moles of formic acid were produced. Aerobic conditions favoured formation of 2,3-butanediol and acetoin, oxidation of the substrate, and formation of acetic and butyric acids, but greatly depressed the amount of glycerol and lactic acid formed. In alkaline media (pH 7.5), acids were formed at the expense of the diol and glycerol.

Introduction

More than 40 years ago Desmots (2) showed that *Bacillus subtilis* produced acetoin when grown on a carbohydrate-rich medium. Later Lemoigne (11) found that this species yielded 2,3-butanediol under these conditions and that the acetoin was formed by bacterial oxidation of the diol. Later work by Friedemann (3) did not agree with these results. He grew two strains of *B. subtilis* on a medium containing glucose, peptone, and meat extract, buffered to an initial pH of 7.6 with sodium phosphate, and found that 70% of the glucose was fermented to lactic acid, the other products being formic acid, acetic acid, and ethanol; the analyses did not allow for any appreciable amount of 2,3-butanediol.

It is not evident whether this disagreement was due to the fact that different strains of *B. subtilis* were used or to some differences in experimental conditions. The latter explanation is supported by some recent experiments of Gunsalus (7). He found that Ford's strain of *B. subtilis* fermented 70 to 90% of the glucose to lactic acid, when in a medium with a low thiamin content, while addition of thiamin lowered the yield to 50% and caused 2,3-butanediol to be formed in about 26% of the theoretically possible amount.

A preliminary report from these laboratories (14) has shown that the *B. subtilis* fermentation is even more complex than the above studies indicate. In addition to 2,3-butanediol and lactic acid, glycerol was discovered to be a major product under certain conditions. This paper presents the complete carbon balance data in support of these claims and outlines a few of the factors that influence the course of the fermentation. Proof of the identity of the products has been established by their isolation and subsequent identification as crystalline derivatives.

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as Paper No. 27 on the Industrial Utilization of Agricultural Wastes and Surpluses and as N.R.C. No. 1330.

[2] *Biochemist, Bacteriologist, and Mycologist, respectively.*

Experimental

Organism and Media

The strain of *Bacillus subtilis* (N.C.T.C. 2586) used was obtained from Dr. A. G. Lochhead, Central Experimental Farm, Ottawa; it was number 246 in his collection. It is now carried as number 9789 in the American Type Culture Collection.

The media used are listed in Table I; Difco yeast extract and meat extract, SMACO vitamin-free casein hydrolysate, and Merck's U.S.P. synthetic thiamin were standard constituents. All other chemicals were of Analytical Reagent grade. When high concentrations of phosphates were used the salts were sterilized separately from the organic materials.

The inoculum was cultured on nutrient broth at 30° C. for 24 hr. and added in 3% concentration.

Analysis of Cultures

The carbon dioxide and hydrogen formed when the organism was grown under a stream of oxygen or nitrogen was accurately determined. The nitrogen was first purified by passing it over freshly reduced copper at 500° C. and then soda-lime, while the oxygen was passed over soda-lime only. The gas, swept from the fermenting solution, was dried by passing through a trap cooled by dry ice, and a calcium chloride tower. It then passed, in order, through a Nesbitt bulb filled with Ascarite, a combustion tube filled with wire-form cupric oxide (500° C.) and two Schwartz tubes filled with anhydrous magnesium perchlorate. The carbon dioxide was measured by the increase in weight of the Ascarite, while the hydrogen was weighed (as water) in the first magnesium perchlorate tube. The Nesbitt bulbs were weighed daily; when the carbon dioxide evolution had practically ceased dilute sulphuric acid was added to kill the organism and release the bound carbon dioxide. Therefore, the carbon dioxide formed from the carbohydrate is equal to the total carbon dioxide obtained less that added to the medium as calcium carbonate.

The contents of the cold trap was added to the fermented solution and the bacteria and proteins were precipitated together by tungstic acid and centrifuged out. The total carbon content of this precipitate less that of a similar precipitate obtained from the original medium represents the carbon assimilated.

The supernatant liquid was then made up to a definite volume and analysed for fermentation products. Ethanol was estimated by dichromate oxidation of the neutral volatile products, a correction being made for the acetoin present. The other products were determined in another 100 ml. aliquot. This was first adjusted to pH 8.5 and extracted continuously for two days with ether under controlled conditions. The extract was made to 100 ml. with water, after evaporation of the ether, and used for the determination of acetoin and 2,3-butanediol. The residual aqueous solution was acidified to pH 1.5 to 2.0 with sulphuric acid and further extracted for one day with

ether. After addition of 20 ml. of water the ether was evaporated and the extract titrated to the phenolphthalein end-point with standard sodium hydroxide to determine the total volatile acids. It was then made to a volume of 100 ml. for use in the subsequent estimation of each organic acid. The extracted aqueous residue was neutralized, cleared with neutral lead acetate, made up to 250 ml., and saved for the determination of glucose and glycerol.

Acetoin and glucose were determined by a copper reduction method using the Shaffer–Somogyi reagent (18). Glycerol and 2,3-butanediol were determined by their periodate reducing values (16), the former being corrected for the glucose present and the latter for acetoin. The extractions were controlled so corrections could be made for the small amount of glycerol extracted with the 2,3-butanediol (10%) and for the 2,3-butanediol not extracted (3%). Lactic acid was estimated by Friedemann and Graeser's method (4) and succinic acid by a modification of Moyle's method (13). Volatile fatty acids were determined following Hillig and Knudsen's directions (9), the formic acid being estimated in the distillates by Lazzari's method (10).

Isolation and Identification of Fermentation Products

Five litres of a medium containing glucose (3%), yeast extract (0.5%), and calcium carbonate (0.5%) was fermented at 30° C. until all the glucose was consumed (nine days). It was then filtered through diatomaceous earth and separated into alcohol, diol, acid, and glycerol fractions by distillation and ether extraction as described in the preceding section on analysis. The acid fraction was divided into volatile and non-volatile acids by steam distillation. The glycerol fraction was concentrated to 300 ml. and made into plaster, which was broken up and extracted with ether for three days to remove the glycerol. The crude glycerol, ethanol, and 2,3-butanediol were each purified by fractional distillation and then identified as crystalline derivatives. The lactic acid was purified and identified as lithium lactate. The volatile acid was divided into two parts; one was converted directly into the *p*-bromophenacyl esters while the other was refluxed with mercuric sulphate (to destroy the formic acid) and steam distilled. The last 30% to distill was converted to the *p*-bromophenacyl ester and recrystallized from methanol–water and petroleum ether to obtain the pure *p*-bromophenacyl ester of acetic acid. The mixed *p*-bromophenacyl esters obtained from the other part of the total volatile acid fraction yielded the pure butyric acid derivative on recrystallizing from methanol–water and the formic acid derivative on recrystallizing from petroleum ether (b.p 60° to 80° C.).

Results

It was found (see Table I) that the composition of the medium and the experimental conditions have a marked effect on the course of glucose dissimilation. Production of lactic acid rather than 2,3-butanediol is favoured by a high pH or a high protein content of the medium, while 2,3-butanediol and acetoin formation is favoured by the presence of oxygen, a low pH, and

to a slight extent by thiamin. In all these experiments 90 to 100% of the glucose was accounted for, the other products being ethanol, glycerol, and volatile acids. The most significant of these results are shown in Tables II and III.

TABLE I

GLUCOSE DISSIMILATION UNDER VARIOUS CULTURAL CONDITIONS

Medium No.	Conditions				Millimoles per 100 millimoles of glucose fermented		Ratio
	pH		Temp. °C.	Gas used in aeration	2,3-Butanediol*	Lactic acid	Diol*/ lactic acid
	Initial	Final					
1	6.8	6.2	30	O_2	66.98	1.77	37.80
	6.8	6.2	30	N_2	58.04	19.96	2.92
2	7.6	6.0	30	None	20.43	98.50	0.21
	7.6	6.4	37.5	None	15.76	69.25	0.23
3	6.2	5.8	30	None	56.16	39.13	1.43
	7.6	6.8	30	None	36.16	53.08	0.68
	7.6	6.8	37.5	None	31.25	57.60	0.55
4	7.6	6.8	37.5	None	33.98	53.40	0.64

Media

1. Yeast-extract–glucose (glucose, 3%; yeast extract, 1.0%; calcium carbonate, 1.0%).

2. Friedemann's (glucose, 1.0%; peptone, 1.0%; meat extract, 0.3%; disodium hydrogen phosphate, 1.5%).

3. Low-nitrogen medium (glucose, 3.0%; vitamin-free casein hydrolysate, 0.1%; disodium hydrogen phosphate, 1.0%).

4. Low-nitrogen medium + thiamin (above + thiamin (12 micrograms per ml.)).

* *Includes millimoles of acetoin.*

The effect of oxygen on the fermentation is shown in Table II. Under anaerobic conditions 2,3-butanediol and glycerol are the main products at pH 5.8 to 6.2, but appreciable amounts of lactic acid, ethanol, and formic acid are found. Oxygen favours formation of the four-carbon products but either depresses the formation of lactic acid and glycerol or oxidizes them. This organism probably oxidizes 2,3-butanediol to acetoin and it is possible that the acetic and butyric acids formed, and the excess carbon dioxide evolved, in the presence of oxygen, come from the oxidation of glycerol or lactic acid.

The effect of pH is shown in Table III. All acids are produced more actively in alkaline solutions at the expense of 2,3-butanediol and glycerol. The production of ethanol is also favoured by a high pH.

Only traces of glycerol were found in the experiments (Table I) in which media with a high pH and protein were used.

Since the methods of analysis are not completely specific the identity of the products was proved by isolating them and forming crystalline derivatives,

TABLE II

DISSIMILATION OF GLUCOSE UNDER AEROBIC AND ANAEROBIC CONDITIONS

Product	Millimoles per 100 millimoles of glucose dissimilated	
	Grown under nitrogen	Grown under oxygen
2,3-Butanediol	56.38	33.35
Acetoin	1.66	33.63
Glycerol	39.91	3.66
Ethanol	12.88	7.38
Lactic acid	19.96	1.77
Formic acid	5.56	1.03
Acetic acid	Doubtful	4.99
n-Butyric acid	0.35	1.24
Carbon dioxide	122.00	207.58
Carbon assimilated	11.09	34.00
Hydrogen	Nil	Nil
Carbon accounted for	96.6%	93.0%
Glucose dissimilated (8 days)	100.0%	98.9%
O/R index	1.04	2.24*

Medium: *Glucose, 3%; yeast extract, 1%; calcium carbonate, 1%.*

Conditions: *Cultured at 30° C. using 300 ml. of medium in a 1000 ml. Erlenmeyer flask. The gases were bubbled through at the rate of about 100 ml./min. The pH was kept at 6.8 to 6.2 by the carbonate.*

* *This high value indicates oxidation of substrates.*

TABLE III

EFFECT OF pH ON THE DISSIMILATION OF GLUCOSE

Product	Millimoles per 100 millimoles of glucose fermented	
	Grown at pH 6.2 to 5.8	Grown at pH 7.6 to 6.8
2,3-Butanediol	56.16	36.16
Acetoin	Trace	Trace
Glycerol	26.28	16.39
Ethanol	18.24	28.70
Lactic acid	39.13	53.08
Succinic acid	Trace	5.05
Formic acid	9.97	30.14
Acetic acid	Doubtful	3.98
n-Butyric acid	Doubtful	2.76
Carbon dioxide (calc.)	130.56	101.01
Carbon accounted for	100.0%	97.0%
Glucose dissimilated (4 days)	73.7%	68.2%

Medium: *Glucose, 3%; potassium dihydrogen phosphate, 0.5%; dipotassium hydrogen phosphate, 0.6%; magnesium sulphate heptahydrate, 0.02%; casein hydrolysate, 0.1%.*

Conditions: *Grown at 30° C. without aeration; the pH was measured with a glass electrode every 8 to 10 hr., and adjusted with N sodium hydroxide.*

which were identified by their melting points; in each case a mixed melting point determination with an authentic sample was carried out. Thus glycerol was identified as the tribenzoate and tri-*p*-nitrobenzoate (m.p. 192° C.), ethanol as the *p*-nitrobenzoate (m.p. 57° C.), 2,3-butanediol as the bisphenylhydrazone of diacetyl (m.p. 245° C.) after oxidation (12), and formic, butyric, and acetic acids as the *p*-bromophenacyl esters (m.p., 140°, 63°, and 85° C., respectively). The lithium lactate was identified by permanganate oxidation (4); one mole of this salt yielded 0.96 mole of acetaldehyde. The 2,3-butanediol had physical properties intermediate between those of the *levo*- and *meso*-isomers (b.p., 181° to 182° C. (760 mm.), $[\alpha]_D^{26} = -5.0°$, and refractive index = 1.4340 at 26° C.), and is believed to be a mixture of the two forms in approximately equal amounts.

Discussion

It is apparent from the results of this study and those of other investigators that the *Bacillus subtilis* fermentation is very complex. In contrast with the classic alcoholic and lactic acid fermentations, which are relatively stable, minor environmental changes markedly affect the course of the fermentation. Certainly the use of different media and experimental conditions is partly responsible for the controversial nature of the results obtained by different investigators. The use of different strains of the organism may have also influenced the results.

This 2,3-butanediol fermentation differs from other well known ones (1, 8, 17) by not producing molecular hydrogen. Instead the hydrogen made available by production of one mole of 2,3-butanediol from one mole of glucose is used to reduce one mole of triose to glycerol. Thus under the most favourable conditions the dissimilation of glucose approaches the over-all reaction:—

$$3C_6H_{12}O_6 \longrightarrow 2\,CH_3.CHOH.CHOH.CH_3 + 2CH_2OH.CHOH.CH_2OH + 4\,CO_2$$

Actually under the best conditions realized by us (Table II) about 10% of the glucose is fermented to lactic acid and 5% to ethanol. In addition the yield of glycerol is only about 70% of what it should be compared to the 2,3-butanediol; this may be because it is slowly attacked by the organism.* However, it is to be expected that the ideal fermentation described by the above equation may be realized experimentally by using the right organism under appropriate conditions.

Any glucose dissimilation that makes hydrogen available might be expected to yield glycerol as a product. For example, acetic acid and glycerol might be formed according to the equation:

$$3C_6H_{12}O_6 + 2H_2O \longrightarrow 4CH_2OH.CHOH.CH_2OH + 2CH_3COOH + 2CO_2$$

and indeed it has been shown by Gayon and Dubourg (5, 6) as well as by Nelson and Werkman (15) that certain heterofermentative lactic acid bacteria

**Recently we have obtained equimolecular proportions of 2,3-butanediol and glycerol, using other strains.*

dissimilate part of the glucose in this way*, although most of the glucose is fermented to lactic acid. However, it is possible that organisms exist that dissimilate practically all the glucose according to this equation.

It is quite probable that this glycerol fermentation may be of industrial importance if active organisms giving high yields are obtained. Although the fermentation will require close control, it should be more satisfactory than glycerol production from yeast using the sulphite or alkaline process.

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** Dr. I. C. Gunsalus has kindly called our attention to this work, which we had overlooked in a previous publication (14).*

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